PREDICTION OF TYPE-1 DIABETES

EVALUATION OF ASSAYS FOR β-CELL ANTIBODIES

PREDICTIE VAN TYPE-1 DIABETES

EVALUATIE VAN BEPALINGEN VOOR β-CEL ANTISTOFFEN

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Cover Illustration:
The cover illustrates the theoretical efficacy of antibody screening for diabetes prediction, derived from the studies described in chapter 3 of this thesis. Of 1000 individuals, five tested positive for ß-cell antibodies. Two of these (depicted in green) will develop type-1 diabetes in the future, while the other three (false positives - depicted in red) remain unaffected throughout life. In addition, one individual that will develop diabetes in the future escapes from detection by antibody screening (upper right corner of front-page).
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PROEFSCHRIFT

Ter verkrijging van de graad van doctor aan de Erasmus Universiteit Rotterdam op gezag van de rector magnificus Prof. Dr. P.W.C. Akkermans M.A.
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Het werk beschreven in dit proefschrift werd uitgevoerd op de Faculteit Geneeskunde en Gezondheidswetenschappen van de Erasmus Universiteit en het Academisch Ziekenhuis Rotterdam op de afdelingen Klinische Genetica en Kindergeneeskunde. Het praktisch werk dat wordt beschreven en het drukken van dit proefschrift zijn mede mogelijk gemaakt door de financiële steun van het Diabetes Fonds Nederland, Zorgonderzoek Nederland, het Child Health en Wellbeing fund, de stichting Klinische Genetica Rotterdam en Novo Nordisk Farma BV.

Ik wil best een eind meegaan
met die, die en die
en ook wel met deze of gene:
maar steeds onder voorbehoud
van de vrijheid
om, bijvoorbeeld, wanneer iedereen zwijmelt
over sexus, plexus en nexus te zeggen:
geef mij Suske en Wiske maar,
of, waar het vrije vers alleen troef is,
met een sonnetje uit te komen

(c.q. vice versa)

(Uit: Cees Buddingh' Deze kant boven)

Contents

Scope of this thesis

Type-1 diabetes mellitus is an autoimmune disease. The clinical manifestation is the end-point of a subclinical process that destroys the insulin producing β-cells in the islets of Langerhans (prediabetes). Prediabetes may last months to years and theoretically gives the unique opportunity for prevention of the disease. This requires the availability of an effective therapy and the possibility to reliably identify individuals who are eligible for such a therapy.

In order to develop intervention strategies, studies on the pathogenesis of Type-1 diabetes are required. At the clinical manifestation of the disease β-cell autoimmunity has been present for years. As a consequence disease-initiating events will have disappeared and the immune response and pathophysiology of the β-cells may have changed due to the ongoing destruction process. Thus, studies of the prediabetic phase are a prerequisite. The identification of those who are at high risk to develop the disease is therefore an important key to further studies on the pathogenesis of Type-1 diabetes. In addition, clinical trials of potential prevention strategies require the development of reliable techniques to identify such individuals.

Using a combination of genetic markers and autoantibodies against ß-cell antigens, prediction is possible in first-degree relatives of patients with Type-1 diabetes. For relatives baring high genetic risk markers and being positive for more than one ß-cell autoantibody the risk to proceed to diabetes exceeds 60%. However, the majority of new cases of Type-1 diabetes occur in the general population and it is not clear whether prediction in the general population is as efficient as in first degree relatives.

This thesis aims to

- Decipher the natural course of ß-cell autoantibodies and their relation to disease progression in established diabetes and prediabetes
- Improve knowledge on the applicability of antibodies for the prediction of Type-1 diabetes in the general population
- Improve the performance of screening by technical evaluation of assays to detect GAD- and IA2-antibodies in serum.

In chapter 2 of this thesis autoantibodies against ß-cell antigens are used as a tool to study the natural course of ß-cell autoimmunity both shortly after diagnosis of Type-1 diabetes and in first-degree relatives of patients with Type-1 diabetes. The observations are correlated to clinical parameters of disease progression.

The feasibility of extrapolation of data on diabetes prediction obtained in family based populations is described in chapter 3. In addition, the application of antibodies as a diagnostic marker in adult onset diabetes is described.

Chapter 4 describes the technical evaluation of ß-cell antibody assays and provides guidelines to establish reference values for GAD-antibodies.

The practical work described in this thesis has contributed to improve prediction of Type-1 diabetes mellitus and may form a basis for further standardisation of antibody testing.

Chapter 1
General introduction

1.1. Diabetes mellitus

1.1.1. The diabetic syndrome

Diabetes mellitus is a syndrome of disturbed glucose metabolism characterised by hyperglycaemia. Several pathogenetic processes, ranging from autoimmune destruction of the insulin producing cells in the pancreas to resistance to insulin action in peripheral tissues, are involved in the pathogenesis of diabetes. Deficient insulin production and secretion and insulin resistance may co-exist in one patient and it is often difficult to distinguish the primary cause of hyperglycaemia [1, 2].

The spectrum of clinical manifestation of diabetes may vary widely. Most young cases will present with characteristic symptoms of hyperglycaemia, such as thirst, polyuria, blurred vision, weight loss or keto-acidotic coma. In other cases these symptoms may develop gradually and therefore go unnoticed for a long time. These patients may present with vague symptoms or hyperglycaemia may be detected in routine check-ups.

People suffering from diabetes are at risk to develop specific acute complications like diabetic ketoacidosis, hyperglycaemic hyperosmolar coma and hypoglycaemia. At long disease duration over 70% of the patients will develop at least one of the chronic complications including vascular, renal, ophthalmic and neurological disorders [3-5]. Such long-term complications may be significantly delayed or abolished by normalising blood glucose levels [6-8]. This requires intensive individual treatment and significant changes in lifestyle.

The emotional and social impact of diabetes may cause significant burden in patients and their family. In addition, diabetes has serious economic impact. In 1992 the estimated costs of diabetes in the United States were between $85 and $92 billion, two thirds of which resulted from lost productivity due to hospital admissions or death [9]. The world-wide prevalence of all forms of diabetes is expected to increase

with 35% to 300 million people by 2025 [10]. This estimate excludes children younger than 20, while these children are likely to require most of the healthcare resources, due to their lifelong treatment and high risk to develop chronic complications. Increased knowledge on glucoregulation and the development of new forms of insulin and oral hypoglycaemic drugs have substantially improved quality of life of patients with diabetes and has minimised the rate of acute complications. However, the Diabetes Control and Complications Trial (DCCT) and United Kingdom Prospective Diabetes Study (UKPDS) have demonstrated that even very intensive glucoregulatory treatment can not completely abolish long-term microvascular complications [6-8, 11]. This urges to develop and implement preventive measures for diabetes. Political and organisational decisions on this subject need to be taken now, since implementation of preventive measures in healthcare systems will take years.

1.1.2. Classification of diabetes

Four major variants of diabetes can be discriminated. In 1997 the American Diabetes Association provided a classification of these variants based on the proposed aetiology of the disease (Table 1.1.) [12]. Although classification may be important to assess the prognosis of the disease and for research purposes, treatment of diabetes is mostly based on clinical observations.

Type-1 diabetes (table 1.1. category I) is caused by the loss of insulin producing β-cells and is lethal if not treated with exogenous insulin injections. Biochemically this type of diabetes is characterised by low or undetectable endogenous insulin levels, severe hyperglycaemia and ketoacidosis if not treated. Type-1 diabetes may occur at any age with acute symptoms like thirst, polyuria, weight loss and ketoacidosis. Occasionally type-1 diabetes presents with moderate hyperglycaemia that can change to severe hyperglycaemia in the presence of stress.

In contrast to previously applied terminology, where type-1B diabetes referred to autoimmune polyglandular syndrome, the etiological classification refers to diabetes type-1B as hyperglycaemia caused by non-autoimmune mediated ß-cell destruction.

Table 1.1. Etiological classification of diabetes mellitus (12)

I	Type-1 diabetes (β-cell destruction, usually leading to absolute insulin deficiency) A: Immune mediated B: Idiopathic
II	Type-2 diabetes (may range from predominantly insulin resistance with relative insulin deficiency to a predominantly secretory defect with insulin resistance)
III	Other specific types ▪ Genetic disorders of β-cell function ▪ Genetic defects in insulin action ▪ Diseases of the exocrine pancreas ▪ Endocrinopathies ▪ Drug- or chemical- induced ▪ Infections ▪ Uncommon forms of immune-mediated diabetes ▪ Other genetic syndromes associated with diabetes
IV	Gestational diabetes mellitus (GDM)

Type-2 diabetes (table 1.1. category II) is caused by an interaction between insulin resistance and failure of the ß-cells to compensate for the increased insulin requirement (1). Biochemically type-2 diabetes is characterised by hyperglycaemia and increased plasma insulin levels. Ketoacidosis seldom occurs in these patients. The risk to develop type-2 diabetes increases with age, obesity and lack of physical activity (13). Weight reduction and/or pharmacological treatment may improve insulin resistance, but insulin levels are seldom restored to normal. Treatment with exogenous insulin may be needed to achieve sufficient glucoregulation to prevent long-term complications, but is usually not crucial to survive. The clinical manifestation of type-2 diabetes may be atypical; hyperglycaemia often develops gradually and may in early

stages not be severe enough to cause symptoms. Occasionally, non-symptomatic hyperglycaemia may be present for a long time in sufficient degree to cause pathological and functional vascular or neurological complications and clinical manifestation may occur through neuropathy, nephropathy or retinopathy [14, 15].

Other specific types of diabetes mellitus (table 1.1. category III) include those types of which the underlying disease or process has been identified. The discrimination between type-1 and type-2 diabetes and the other specific types made in the 1997 classification is arbitrary since knowledge on the pathogenesis of most types of diabetes is limited. It is conceivable that the discovery of new genetic or environmental factors playing a role in the pathogenesis of type-1 or -2 diabetes will cause a major shift in the current classification.

Gestational diabetes is carbohydrate intolerance diagnosed or becoming manifest during pregnancy. Although many patients with gestational diabetes will not develop diabetes later in life, the risk to develop type-1 or type-2 diabetes post-partum, even several years after pregnancy, is increased in these women [16, 17].

1.2. Type-1 diabetes mellitus

Type-1 diabetes mellitus is an autoimmune disease that destroys the ß-cells. The aetiology and pathogenesis of type-1 diabetes mellitus are not fully understood. In more than 85% of the patients antibodies to ß-cell antigens can be detected in the circulation at clinical diagnosis [18-23]. In addition, the distribution of HLA-DR and DQ alleles among patients with type-1 diabetes is significantly different from the general population [24-27]. The subdivision of type-1 diabetes in immune mediated and non-immune mediated forms (table 1.1. category I) is arbitrary. It is not clear whether this subdivision is based on the true existence of different forms of type-1 diabetes or on failure to detect autoimmunity, due to our limited understanding of the underlying

immune processes. The work described in this thesis is based on an autoimmune pathogenesis of type-1 diabetes.

1.2.1. Epidemiology

In The Netherlands the annual incidence of type-1 diabetes among children younger than 14 years was 14.3 per 100,000 in the period from 1993 – 1995. Compared to earlier studies this is an increase of 13% over a five years period [28, 29]. In addition, the clinical manifestation has shifted to younger ages [29, 30]. Both observations are confirmed by studies in other countries and since the changes occur over a relative short period of time they must be attributed to environmental factors (paragraph 1.2.2.5.) [1, 31-35]. Other evidence for the role of environmental factors in the pathogenesis of type-1 diabetes comes from the reports of seasonal patterns in the incidence; the highest incidence being in late winter months and the lowest incidence from April to July [31, 36-38]. Some authors favour the hypothesis that increased prevalence of infectious diseases in winter months plays an important role [39-42]. However, it is generally assumed that the time between initiation of type-1 diabetes and clinical manifestation lasts months to years (paragraph 1.2.2.2.). It is more likely that the increased incidence during wintertime is due to the fact that infections are accompanied by increased physiological stress leading to higher insulin requirements rather than being the initiating agent for autoimmunity. Alternatively, it has been reported that patients with diabetes are more often born in spring and early summer than during winter months, suggesting that intrauterine or neonatal exposure to infectious agents during winter months may initiate ß-cell autoimmunity [42-45].

Several nation-wide registries have been combined to establish the world-wide incidence of type-1 diabetes. These international networks show large differences in incidence rates across populations [33, 46]. In general, a north to south gradient of incidence exists; Finland having the

highest incidence (35.2 per 100,000 per year from 1987-1989) [47] and Greece, Italy and the Balkan states having the lowest incidence (varying from 6.5 in Latvia to 7.9 per 100,000 per year in Croatia and Italy) [33, 48]. A remarkable exception to this gradient is Sardinia, where the incidence is similar to the Finish rate [49]. Using a migration study Muntoni and colleagues demonstrated that the increased incidence in Sardinia could be attributed to the genetic background of the population. Children born from Sardinian parents in the Lazio region retained the high Sardinian incidence of type-1 diabetes, whereas children born from one Sardinian parent in the Lazio region had half the incidence rate of the Sardinians (still double the rate of the indigenous population) [50].

Additional evidence for the role of genetics in the pathogenesis of type-1 diabetes comes from the observation that the incidence is 5-10 fold increased in first degree relatives of patients with type-1 diabetes compared to the general population [51]. However, of all new cases diagnosed only 10% has a first degree relative with the disease. Paragraph 1.2.2.4. will discuss the role of genetics in the pathogenesis of type-1 diabetes in detail.

All epidemiological studies presented here describe onset of type-1 diabetes in children. It is of importance to mention that type-1 diabetes is not solely a disease of childhood and that as much as 30-50% of new cases of type-1 diabetes may present in adults [52-61]. These forms of adult onset diabetes are often initially treated as type-2 diabetes but rapid loss of c-peptide, proneness to develop ketoacidosis and the presence of autoantibodies in serum indicate that they represent a slow progressive form of type-1 diabetes. This form of diabetes is designated Latent Autoimmune Diabetes of Adults (LADA) [60]. With increased possibilities to detect autoimmune phenomena, studies on the prevalence of this form of diabetes in the general population have been initiated (chapter 3.3).

1.2.2. Aetiology and pathogenesis

1.2.2.1. Histopathological studies

The hallmark of type-1 diabetes is selective destruction of the ß-cells in the islets of Langerhans. Post-mortem studies of patients with type-1 diabetes revealed that the other endocrine cells constituting the islets of Langerhans, glucagon, somatostatin and PP-cells, are virtually unaffected, although their distribution may be slightly altered [62, 63]. Occasionally a few remaining ß-cells can be found even after 9 years of clinical diabetes [63, 64].

Only limited data on histology of on human pancreas at onset of the disease are available, since fortunately only few people die at onset of the disease. In addition to ß-cell loss, the histopathological picture of pancreases of recent onset diabetes patients is characterised by a florid inflammatory process of islets of Langerhans [65-70], often referred to as insulitis. Three kinds of islets can be discriminated at onset:

- Islets depleted from ß-cells, but with a normal distribution of glucagon, somatostatin and PP-cells, similar to the islets observed in long-standing diabetes.
- Islets with some remaining ß-cells and massive infiltration with immune cells.
- Apparently unaffected islets with normal insulin-, glucagon-, somatostatin- and PP-cells.

The heterogeneity in the histopathology indicates that islet cell destruction is a dynamic process that is far from complete at time of diagnosis and does not affect all islets at a time. This suggests the existence of heterogeneity in the constitution of the islets of Langerhans, or at least differences in functionality, that render some islets more susceptible for autoimmunity than others [71].

The histopathological descriptions of insulitis and the selective nature of ß-cell destruction have lead to the concept of a gradual process of ß-cell destruction that may take years to destroy such an amount of ß-cells

that clinical symptoms occur [65]. This concept is in keeping with observations in NOD mice, a spontaneously diabetic mouse obtained from twenty generations inbreeding of CTS mice [72]. In these mice insulitis occurs several weeks prior to onset of hyperglycaemia (paragraph 1.2.2.3.1.). Insulitis seems to be highly regulated and can be subdivided into two phases. In the first phase, called peri-insulitis, antigen presenting cells and T-cells build up around the islets of Langerhans but there is no detectable infiltration or ß-cell destruction [73-76]. In the second phase a more aggressive pattern is observed; T-cells infiltrate the islets and start producing cytotoxic cytokines. In this phase massive ß-cell destruction occurs [77, 78]. In addition to the changes in the islets of Langerhans, autoantibodies directed to ß-cell antigens are detected in the circulation and changes in the insulin response to a glucose challenge may occur several years before clinical onset in mice and humans [77, 79-86].

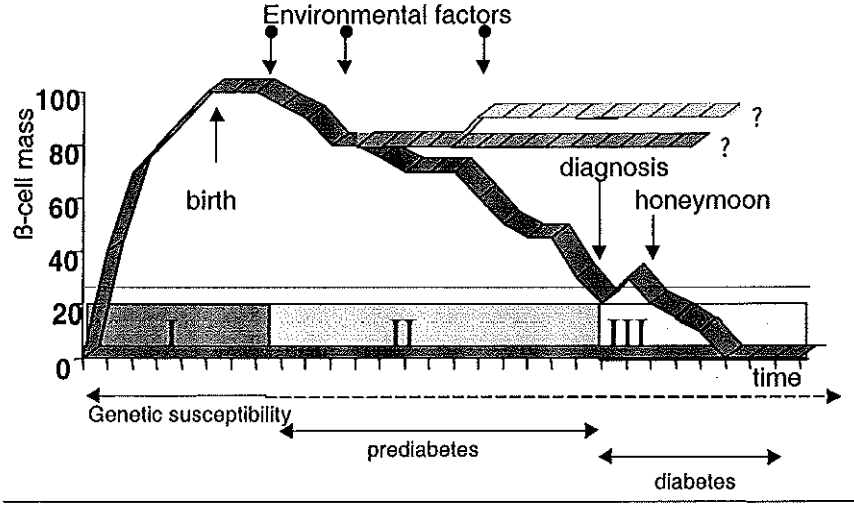
1.2.2.2. Prediabetes

In both humans and NOD mice the development of type-1 diabetes has three distinct stages.

- A period of genetic susceptibility to the disease (figure 1.1. phase I), further explained in paragraph 1.2.2.4.
- A period of leucocytic infiltration of the islets of Langerhans and selective ß-cell destruction. This period, referred to as prediabetes, may last months to years in humans. (figure 1.1. phase II). ß-cell destruction occurs asymptomatic, but, as a consequence of ß-cell specific immune activation, autoantibodies to ß-cell antigens occur in the circulation.
- Overt type-1 diabetes starting with clinical manifestation once approximately 70% of the initial ß-cell mass has been destroyed. After initiation of insulin therapy a period of insulin independence may occur (the honeymoon). This is most likely the result of

restoration of insulin sensitivity, due to the correction of hyperglycaemia. In addition, insulin therapy may have a beneficial effect on the remaining ß-cells or even stimulate ß-cell regeneration (87). Since the destruction of ß-cells will continue until (virtually) no ß-cells are left, this period will come to an end within weeks or at maximum a few months.

Figure 1.1: A model of ß-cell decline during (pre-)diabetes



It is not clear whether all patients entering into stage II proceed to clinical diabetes. Possibly some individuals are able to correct the autoimmune attack thus halting ß-cell destruction and remaining asymptomatic (indicated with question marks in figure 1.1.). This may represent individuals who are tested positive for ß-cell antibodies but who never develop diabetes. Evidence is accumulating that ß-cell regeneration is possible and these individuals may eventually end up with normal ß-cell mass.

1.2.2.3. Pathogenesis

1.2.2.3.1. Of mice

Strong evidence, both in humans and rodents, suggest that T-cells are the major contributors to the pathogenesis of type-1 diabetes. In NOD mice, the transfer of diabetes into irradiated recipients involves the participation of both CD4+ MHC class II restricted (helper) and CD8+ MHC class I restricted (cytotoxic) T-cells [88-90]. The important role of T-helper (Th) cells in the pathogenesis of diabetes may reflect a critical role for immunoregulatory processes in the pathogenesis of type-1 diabetes.

Table 1.2. Th1 and Th2 cytokine profiles in mice

	Th1	Th2
Differentiation initiated by	IL12	IL4
Effect on ß-cell destruction	Promotion	Protection
Cytokine production pattern	IFNγ, TNFß, IL2	IL-4, IL-5, IL10, IL13
Antibody pattern	IgG2	IgG1, IgE, IgA
Macrophage function	Promotion	Inhibition

In mice, the Th population can be divided into Th1 (aggressive) and Th2 (regulatory) cells (Table 1.2.) [91]. Th1 cells are thought to be the initiator of cytotoxic T-cell mediated ß-cell destruction, while Th2 lymphocytes prime the immune response in the humoral direction and are thought to have a protective effect in NOD mice [90, 92-94]. Some authors report the existence of "peri-insulitis", a non aggressive form of insulitis in which the islets of Langerhans are surrounded by lymphocytes, but no invasion of the islets and ß-cell destruction occurs. Peri-insulitis seems to be mediated by Th2 lymphocytes and progression to diabetes does not occur in the absence of Th1 cytokines [74, 90]. Indeed, the incidence of IDDM in NOD mice treated with the "Th2 cytokine" IL-4 or with blocking antibodies against "Th1-cytokines" is decreased, whereas treatment with "Th1-cytokines" leads to increased ß-cell destruction [89] (table 1.2.). Thus, the rate of progression to IDDM

in NOD mice may be mediated through a functional balance of Th1 and Th2 cells.

The priming of CD4+ T-cells to Th1 or Th2 responses is thought to be regulated via antigen presenting cells (APCs) and is dependent on the route of entry and concentration of an antigen [91, 95-97]. In NOD mice, macrophages and dendritic cells are the first cells to enter the islets of Langerhans and abnormal maturation of APCs has been demonstrated, suggesting a key-role in the pathogenesis of the disease [98-101]. Recent studies show that dendritic cells may be considered as a heterogeneous group of immune cells with an immunoregulatory role, which may be primed into either a destructive or protective direction [95, 102]. Jun and colleagues described that depletion of APCs in NOD mice results in prevention of diabetes. This is accompanied by a shift of the immune balance to a Th2 response and decreased T-cell activation. In this study diabetes prevention could be reversed by administration of IL-12 [103]. Additionally, dendritic cells seem to be involved in morphogenesis and function in several endocrine tissues such as the pituitary, gonads, thyroid and islets of Langerhans [104-107]. Thus, dendritic cells and macrophages play an essential role in the pathogenesis of diabetes in NOD mice and may do so by defective maturation and co-operation between ß-cells and dendritic cells or by dysregulation of the balance between Th1 and Th2, resulting in activation of ß-cell-cytotoxic T-cells. An important role is assigned to cytokines, accessory molecules, FAS and FAS-ligand, CTLA4 and adhesion molecules (ICAM-1) to maintain the balance in the immune system [98, 108, 109]. Indeed, various studies have described that in NOD mice diabetes can be prevented by deviating the immune response in the Th2 direction either by cytokine therapies or via administration of high dosages of ß-cell antigens (tolerance induction) [110-115].

Using a model of transgenic NOD mice expressing a T-cell receptor that recognises a ß-cell antigen, Andre-Schmutz and colleagues

demonstrated that the susceptibility to induce diabetes by cyclophosphamide injections decreases with age in these mice. Although this is a highly artificial system, this provides an indication that diabetes is a result of events occurring early in life. Interestingly, they demonstrate that in early infiltrates (peri-insulitis) B-cells are adjacent to the islet-cells and T-cells reside more in the periphery of the infiltrates [109], suggestive for an antigen-presenting role for B-cells early in the pathogenesis of type-1 diabetes in these mice. This hypothesis was confirmed by Falcone and colleagues, who demonstrated that B-cells are required to induce T-cell responses to ß-cell antigens [116].

1.2.2.3.2. And men

Because the pancreas is not accessible for immunological investigation in humans, effector mechanisms must be studied through cells obtained from the peripheral blood (PBMCs). This has the disadvantage that events occurring in the target organ may not be reflected in the periphery and it is conceivable that recruitment of particular types of cells to the pancreas leads to under-representation of these cells in peripheral blood. In addition, it is still controversial if the Th1/Th2 dichotomy found in rodents is applicable to humans [117-124].

Several studies have indicated that human type-1 diabetes is characterised by a shift of circulating T-cells in the CD4 direction [125, 126], although others report the contrary [127]. In addition, increased numbers of T-cells reactive to ß-cells or ß-cell proteins are detected in the peripheral blood. [125, 128-135]. This observation is not exclusive to patients; autoreactive cells can be detected in normal controls as well, indicating that autoreactive T-cells alone do not cause diabetes.

Several ß-cell antigens recognised by autoreactive T-cells have been described. The first diabetes related human T-cell clone identified was reactive to a membrane protein of insulin secretory vesicles [131, 132,

[136]. Since then, several ß-cell reactive T-cell clones have been described. T-cell responses to insulin and glutamic acid decarboxylase (GAD) have been most extensively studied [128, 137]. T-cell responses to GAD and specific peptides derived from GAD are detectable in almost half of the newly diagnosed patients with type-1 diabetes and in 10% of healthy controls [133, 138, 139]. Interestingly, T-cells showing dual recognition of GAD_{65} and the Protein 2C (P2C) of coxsackie B viruses have been detected in patients and in first degree relatives who are at increased risk to develop the disease [140-142]. Several epidemiological studies have suggested that coxsackie and other enteroviral infections play a role in the pathogenesis of type-1 diabetes. The mechanism favoured by most authors (molecular mimicry) is based on structural similarities between viral and ß-cell proteins (paragraph 1.2.2.5.2).

Recently, Durinovic-Bello and Ellis and colleagues demonstrated that T-cell responses to another diabetes associated antigen, insulinoma associated antigen 2 (IA-2), are increased in patients with diabetes [143-145]. An IA-2 epitope was reported to show sequence similarity (but not identity) with VP7, a major immunogenic protein of human rotavirus and with epitopes from other viruses that have been reported to play a role in the pathogenesis of type-1 diabetes [146]. However, with emerging knowledge on human, viral and microbial DNA, RNA and protein sequences, the chance on a similarity hit in one of the world-wide used databases is increasing. Such a hit may be coincidental until functional cross reactivity has been demonstrated.

Thus, several pathways leading to diabetes have been described in animal models and there are several antigens that may play a role in the pathogenesis of type-1 diabetes. Dissecting the mechanism in humans requires follow-up studies of prediabetic subjects, standardisation of T-cell assays and a way to visualise the (immune) processes in the human pancreas. Paragraph 1.2.2.4. and 1.2.2.5. describe genetic and

environmental factors that may play a role in initiation and maintenance of the mechanisms described here.

It is important to realise that the tissue specificity of the autoimmune reaction in type-1 diabetes requires some ß-cell specific factor early in the pathophysiology of the disease. Indeed, early changes in the islet structure of NOD mice preceding insulitis have been described and may be involved in the initiation of ß-cell autoimmunity (J. van Rosmalen, personal communication) [147, 148].

1.2.2.4. Genetic susceptibility

1.2.2.4.1. Genetic linkage and association studies

Table 1.3. Recurrence rate of type-1 diabetes in close relatives of patients

	Relation to patient	Recurrence rate
A	Monozygotic twin	23-50%
	Dizygotic twin	3-10%
	Sibling	1-8%
	Father	1-6%
	Mother*	1-4%
	Nephew / niece	1-2%
B	Sibling sharing 2 HLA haplotypes	6-19%
	Sibling sharing 1 HLA haplotype	3-7%
	Sibling sharing 0 HLA haplotypes	1-3%
C	Lifetime risk in the general population	0.3%

Panel A: recurrence rate regardless HLA-type [149-151]
Panel B: recurrence rate dependent on HLA-type [152]
Panel C: basline risk in the general Dutch population [29]
* When diabetes is part of polyglandulair autoimmune syndrome (Schmidt's syndrome) the recurrence rate in offspring is 10 – 40%.

Table 1.3. illustrates the recurrence rate of type-1 diabetes in relatives of patients with the disease. In all close relatives this rate is increased compared to the general population – being a strong indicator for genetics to be involved in the pathogenesis. The concordance in monozygotic twins varies from 25 – 50% [150, 153-156], indicating that genetics alone are not sufficient to develop diabetes. Therefore, it is generally assumed that the susceptibility to type-1 diabetes is inherited,

but additional environmental factors are needed to initiate and maintain ß-cell autoimmunity and develop diabetes. Phase I in figure 1.1. illustrates this period of genetic susceptibility in which the ß-cell mass and immune system are apparently normal. However, it can not be excluded that slight aberrations in ß-cell mass or function are already present at birth.

Table 1.4. HLA DRB1 alleles associated with type-1 diabetes

Allele	Susceptible / protective *	residue 57
DQB1*0201	susceptible	ala
DQB1*0302	susceptible	ala
DQB1*0502	susceptible / neutral	ser
DQB1*0303	protective / neutral	asp
DQB1*0301	protective / neutral	asp
DQB1*0602	protective	asp
DQB1*0603	protective	asp

The magnitude of protective or susceptible effects of an allele may differ per ethnic group, depending on the prevalence rate in the general population – therefore no relative risk scores are given in this table.

As demonstrated in panel B of table 1.3. the recurrence rate in first-degree relatives is strongly influenced by the HLA-haplotype. Whole genome scans in affected sib-pair studies have demonstrated that the major proportion of disease clustering in families can be accounted for by shared alleles in or in linkage disequilibrium with the HLA locus on the short arm of chromosome 6 [157, 158]. From mathematical models it was deduced that between 30 and 60% of the genetic susceptibility for type-1 diabetes can be explained by association with genes in the HLA complex [159]. Early studies indicated a strong correlation between HLA-DRB1 and type-1 diabetes, HLA-DR3 and -DR4 conferring susceptibility and HLA-DR2 and -DR5 conferring protection [160]. In later studies the strongest association was found with HLA-DQ. Alleles encoding an aspartic acid at position 57 of the DQB1 locus ($DQ\beta1^{asp57+}$) and alleles encoding an arginine at the DQA1 locus ($DQ\alpha1^{arg52+}$) are associated with protection from and susceptibility to diabetes respectively (table 1.4.) [26, 157, 161-164]. However, strong linkage

disequilibrium between different loci in the HLA-region makes it difficult to determine which gene is primarily responsible for providing susceptibility or protection to type-1 diabetes. Indeed, Zamani and colleagues have described polymorphisms in the HLA-DR gene that show equally high correlations to the disease [165, 166]. The importance of the HLA region in the pathogenesis of diabetes is emphasised by the fact that polymorphisms in the murine homologue of the HLA (class II I-A) are strongly correlated to diabetes in NOD mice [167-169].

In order to identify additional genomic regions that are associated to type-1 diabetes, genetic linkage studies have been undertaken. In such studies the LOD-score is applied as a statistical estimate of the distance between a genetic marker and a disease gene. In general, a marker or gene is assumed to be linked to a disease if the LOD-score exceeds 3.6. At least 14 diabetes associated loci outside the HLA-complex have been identified (IDDM2-15) [158, 170-175], but only one of these meets this criterion (IDDM10, LOD score 4.7) [158, 174, 175] and only a few candidate genes are located in these regions (table 1.5.).

Table 1.5. Candidate genes for type-1 diabetes susceptibility

Locus	chromosome	Candidate gene
IDDM1	6p21	HLA-DQ, TNF-alpha
IDDM2	11p15	INS VNTR
IDDM4	11q13	FGF3
IDDM5	6q25	ESR1
IDDM12	2q33	CTLA4

Interestingly, almost all candidate genes are involved in immunoregulation, ß-cell function or ß-cell development. This observation confirms the hypothesis that type-1 diabetes is not solely a disease of the immune system, but that aberrant function or development of ß-cells must be involved to explain the ß-cell-specificity of the autoimmune process. It is likely that interaction of several genes mount up to cause the onset of type-1 diabetes. Cordell and colleagues investigated the interactions between the IDDM1, IDDM2 and IDDM4

loci. The interaction between IDDM1 and IDDM2 could best be described as multiplicative whereas the IDDM1 and IDDM4 followed a heterogeneity model (exerting their effect through different biochemical pathways) [176].

1.2.2.4.2. Molecular mechanisms

Since only few candidate genes have been described little is known about the mechanisms that confer susceptibly or protection for type-1 diabetes. For the HLA-region and the INS and CTLA4 genes there have been some speculations.

MHC class II molecules are involved in antigen presentation to T-cells by antigen presenting cells (dendritic cells, macrophages). The DQA1 and DQB1 genes code for an αß-heterodimer that forms the antigen-binding cleft of the MHC molecule. The association of diabetes with $DQB1^{asp57-}$ and $DQB1^{arg52+}$ alleles has therefore been a subject of speculation. It is suggested that certain MHC molecules have the capability to bind and present "diabetogenic" peptides, or provide a "diabetogenic" T-cell activation pattern upon presentation of certain ß-cell peptides. Peptide elution studies by Rammensee and colleagues and Reich and colleagues have provided evidence suggesting that for example certain peptides of glutamic acid decarboxylase, a putative antigen in type-1 diabetes, are preferentially presented by non-asp DQß molecules [177, 178]. In addition, some authors have suggested that the peptides presented by susceptibility haplotypes preferentially present peptides that induce a Th1 response [90]. Heterozygous HLA-DR3/4 haplotypes are associated with the highest frequency of diabetes, with DR3/x and DR4/x (where x stands for any DR haplotype except DR3, DR4 or DR2) invoking more modest levels of risk [26, 179-181]. Perhaps one of the susceptibility haplotypes allows for recognition of specific antigens, while the other is associated with dysregulation of normal immune responses. The involvement of MHC class II in the pathophysiology of type-1 diabetes

fits well with the observation of APC involvement in NOD mice (paragraph 1.2.2.3.1.).

IDDM2 represents polymorphisms in the 5' prime flanking region of the insulin gene. The polymorphism arises from a variable number of tandem repeated oligonucleotides. When divided into 3 size classes (the shortest class being class I), class I alleles seem to predispose for type-1 diabetes, while class III alleles have a dominant protective effect (182-184). It has been demonstrated that class III alleles are associated with marginally lower insulin m-RNA levels in the human pancreas and higher expression of insulin mRNA in thymus than class I alleles. It is proposed that the increased insulin expression in thymus facilitates the induction of immunological tolerance (depletion or anergy of insulin reactive T-cells), thus explaining the protective effect of the INS class III allele (185-187). However, experimental evidence for this hypothesis remains to be established (188).

Combined linkage and association studies have revealed evidence for linkage disequilibrium between CTLA-4 and type-1 diabetes (IDDM12) (171, 189-191). CTLA-4 is a candidate gene for diabetes susceptibility since it encodes a T-cell receptor that mediates T-cell apoptosis and is crucial for negative selection of autoreactive T-cells (192-194). CTLA-4 knockout mice develop a lethal lymphoproliverative disease, which is characterised by massive infiltration and destruction of several organs including the pancreas (195, 196). The CD28 gene that is located close to the CTLA-4 gene is another candidate that may alter the immune response (197). Several other examples of animal models involving knockouts of candidate genes resulting in serious immune deviations are known. However, knockouts are all very artificial models and extrapolation to human diseases should be interpreted with care.

Although over 15 genomic regions have been suggested to be associated to type-1 diabetes and several candidate genes have been suggested, the mechanisms that link genetic messages to immunological and

clinical changes in the disease process remain speculative. Moreover, for several candidate regions a lack of concordance between different populations and even within populations exists. This finding is partly secondary to the polygenic nature of the disease. Recently initiated studies using genetically isolated populations have a higher potential to detect disease associated genes than conventional sib-pair analyses, since it is likely that less genes are involved in the disease in these populations [198].

1.2.2.5. Environmental factors

As described in previous chapters, seasonal and regional variation, the continuous increase in the incidence of type-1 diabetes and the low concordance rates in monozygotic twins provide evidence for the role of environmental factors in the pathogenesis of type-1 diabetes. Environmental factors may play a role in the initiation of autoimmunity in genetically susceptible individuals resulting in progression to prediabetes (figure 1.1. phase I to phase II). In addition, environmental factors may maintain or aggravate autoimmunity, resulting in a quicker onset of the disease (shortening of prediabetes, figure 1.1., phase II). Several compelling reports for the role of toxins, food constituents and viral infections exist.

1.2.2.5.1. Chemicals

Acute diabetic ketoacidosis was reported in five men who ingested the rodenticide Vacor® and a 7-yr-old boy died shortly after Vacor® ingestion. Autopsy studies revealed extensive beta cell destruction and islet cell antibodies were detected in most patients. An in vitro study in isolated rat islets revealed that Vacor® preferentially intoxicates ß-cells and toxicity is reduced by treatment with nicotinamide [199-201]. Interestingly, a large diabetes intervention trial using nicotinamide is currently under evaluation (paragraph 1.2.3.2.).

Streptozotocin is another chemical that induces ß-cell cytotoxicity. In several murine models repeated administration of low doses of streptozotocin induces increased plasma glucose levels, accompanied by insulitis. Interestingly, the ability of streptozotocin to induce diabetes is MHC-dependent. Streptozotocin is frequently used to synchronise diabetes onset in NOD mice, thus facilitating intervention studies and studies of the immunological events that lead to diabetes. [202-205].

1.2.2.5.2. Virus infections

A century ago the first report on diabetes following a recent infection with mumps virus was published [206]. Thereafter several other authors reported the isolation of viruses from the pancreas of patients with recent onset diabetes. Several of these viruses appeared to be diabetogenic in mice [207-211]. Of these, enteroviruses are best documented, but mumps, rubella and cytomegalovirus (CMV) have been correlated to diabetes as well [211-215]. In humans enterovirus infections occur frequently and the seasonal variation of infections coincides with the incidence peak of type-1 diabetes in winter months [216]. Attempts to detect a direct epidemiological correlation are hampered by the fact that most individuals have experienced multiple viral infections by the time of clinical diagnosis. In addition, immunologically mediated damage may occur long after the causative viral infection and evidence for the infection may have been cleared by the time of clinical diagnosis. Despite these drawbacks, cross-sectional studies in newly diagnosed patients have demonstrated that diabetes onset is correlated with increased prevalence of antibodies against enteroviruses, particularly of the IgM class [217-225]. Advances in molecular biology have enabled direct identification of viral RNA from blood or tissue. These techniques have the advantage of higher sensitivity than serology and enable direct identification of the virus involved. The results from such studies are concordant with earlier serological studies in that viral infections are

more common in recent onset patients than in control subjects [226, 227]. It is, however, not clear whether the increased prevalence of infections is secondary to ongoing (pre)-diabetes, viral infections precipitate clinical onset of diabetes or that they play a key role in the pathogenesis of the disease.

Evidence for the latter comes from studies by Hyoty and Dahlquist [41, 220, 228]. In a retrospective case-control study they tested maternal blood samples for the presence of (low affinity) enteroviral antibodies. Blood was collected during early pregnancy or at delivery. They concluded that maternal enterovirus infections are a risk factor for childhood diabetes in offspring. From an additional prospective study Hyoty and colleagues concluded that the number of enterovirus infections experienced early in life could be used as a predictor for progression to diabetes. In this study, enteroviral infections were correlated to changes in levels of ß-cell specific autoantibodies (ICA and IAA). However, the correlation was weak and the time-span between the infections and changes in autoantibodies or onset of clinical diabetes varied widely between the children studied [220]. A causative relationship between enteroviruses and diabetes in humans therefore remains to be established.

Experimental studies have clearly demonstrated that enteroviruses have the potential to cause ß-cell damage, both in vivo and in vitro [215, 229, 230]. However, their acute ß-cytolytic effect seems incompatible with the long pre-clinical period of type-1 diabetes. Theoretically, there are at least two different scenarios in which a virus could produce diabetes. The first is a direct assault on the pancreatic islets during the course of an acute viral infection. The full expression of diabetes resulting from ß-cell damage is presumably a rare event. However, limited attacks by ß-tropic viruses leading to subclinical damage of ß-cells may well occur. Such an event could be the trigger for a series of immunological changes resulting in type-1 diabetes. Shortly, viral infection of

pancreatic tissue will lead to increased immunesurveillance and cytokine release in the pancreas. Antigen presenting cells will pick up and present ß-cell antigens to autoreactive T-cells. In the pro-inflammatory milieu of the pancreas this will lead to activation and subsequent T-cytotoxic ß-cell destruction. This cascade may be initiated either by a direct cytolytic effect of the virus on the ß-cells or by a ß-cytotoxic effect of cytokines (IFN-γ, TNFα) that are released due to the ongoing pancreatitis [231].

The second scenario is molecular mimicry. This does not require infection of the pancreas but assumes that a viral protein cross-reacts with a pancreas antigen. At the site of infection APCs will present these mimicking antigens to T-cells which can, once they are activated, infiltrate the islets of Langerhans. There they are in a position to detect islet antigens and directly destroy ß-cells [232]. The existence of several structural similarities between ß-cell auto-antigens and viral proteins, the most prominent of which is the similarity between GAD_{65} and P2C of Coxsackie B viruses, does support the mimicry hypothesis (paragraph 1.2.2.3.2) [141, 218, 233]. However, most studies are based on similarities in the secondary protein structure, neglecting the fact that the tertiary structure may be of higher physiological significance and direct evidence supporting the role of mimicry in the pathogenesis has never been provided. Alternatively, superantigens could be involved in this scenario. Superantigens are foreign (viral) proteins that are able to directly activate T-cells by binding to the Vß-structures in the T-cell receptor. Indeed, Conrad and colleagues reported that only T-cells bearing a particular Vß-domain infiltrated the pancreas, a picture fitting the super antigen hypothesis [234]. However, this observation was based on only two patients and could not be reproduced by independent laboratories.

Both scenarios assume the presence of autoreactive T-cells and attribute a major role to antigen presenting cells. The presence of these

autoreactive cells may be explained by the cryptic epitope concept. Cryptic epitopes are those self-determinants that are not presented in sufficient amounts to induce T-cell tolerance. T-cells specific for these cryptic epitopes may become activated and autoaggressive if the epitopes are presented at higher concentrations. This may not only occur through increased antigen presentation (increased production or upregulation of MHC-class II), but also through altered antigen processing [235]. Both the presence of autoreactive T-cells and altered antigen presentation have been well documented to exist in NOD mice and to a lesser extent in humans (paragraph 1.2.2.3.).

1.2.2.5.3. Dietary factors

The most extensively studied dietary association with type-1 diabetes is the introduction of cow's milk based infant formula early in life, but still the issue is not settled. Several authors have attributed an increased risk for diabetes to the intake of cow's milk [236-241], while others favour the hypothesis that long duration of breast-feeding protects from diabetes [242-244]. Others were not able to establish any link between cow's milk consumption and diabetes [245-247]. The controversy may be explained by the fact that most studies are based on a mother's recall on infant feeding many years later, which may be unreliable or even biased by the knowledge that a child later developed diabetes [248].

Considering all studies together, early introduction of cow's milk formulas is associated with a mildly increased risk for progression to diabetes later in life. Cow's milk contains casein, bovine serum albumin (BSA) and hormonal substitutes including insulin. Homology between BSA and ICA69, a putative antigen in type-1 diabetes, was suggested to play an important role in the pathogenesis of type-1 diabetes [249]. However, later reports concerning cross-reactivity between BSA and ICA69 autoantibodies and antibody formation against BSA in at risk subjects are controversial, part of which might be attributed to technical

difficulties [250-252]. Autoimmunity to ICA69 is not specific for diabetes but occurs in rheumatoid arthritis as well [253], while cow's milk proteins have also been reported to play a role in some forms of rheumatoid arthritis [254]. Rheumatoid arthritis is associated with different MHC types than diabetes and therefore it is unlikely that the same mimicry epitopes of BSA are involved in both diseases. The increased risk on both diabetes and rheumatoid arthritis associated with early introduction of cow's milk in the diet might rather be due to early introduction of foreign protein in the diet than to a specific cow's milk antigen.

Vaarala reported that antibodies to bovine insulin were detectable in children that have been exposed to cow's milk formula early in life. However, the initial response did not recognise the diabetes-associated epitope of human insulin [255].

ß-casein is far more abundant in cow's milk than in breast milk. 37% of cases with type-1 diabetes have antibodies against ß-casein and T-cell reactivity to casein has been detected in 50% of newly diagnosed children with the disease, suggesting that the immune response to casein plays a role [256]. Another intriguing concept is that cow's milk components might exert detrimental effects on gut or systemic immunoreactivity leading to disturbed peripheral tolerance or enhanced susceptibility to viral infections. Upon digestion, several caseins give rise to opoid peptides that bind to receptors on immune cells leading to activation of mast cells or modulation of T-cell and macrophage reactivity. In NOD mice these forms of casein are diabetogenic. Results from a multicentre study performed by Elliott and colleagues strongly support this hypothesis. They described that consumption per capita of the ß-caseins A1 + B, which upon cleavage give rise to peptides with opoid properties [257, 258], strongly correlates to the diabetes incidence. It is very well possible that breakdown of tolerance due to dietary factors consist of a two-step mechanism. Step 1 being destabilisation of the gut

immune system by some biologically active components (opoids) and step two being presentation of antigens that are capable of breaking self-tolerance through molecular mimicry or associated mechanisms.
Other dietary factors that may increase diabetes risk are high coffee consumption and intake of nitrites and nitroso compounds [45, 259-263].

1.2.3. Prediction and prevention

The concept of prediabetes described in paragraph 1.2.2.2 (figure 1.1.) offers the unique opportunity for disease intervention before onset of clinical symptoms. This requires a reliable method for the identification of individuals at increased risk to develop the disease and the development of adequate preventive measures. There are two ethical problems inherent with screening and intervention:

- The introduction of awareness of risk in the general population.
- The possibility of false positive screening resulting in unjust treatment.

According to the position statement of the American Diabetes Association (ADA), there is sufficient data to warrant type-1 diabetes intervention, but only in the context of defined clinical studies with institutional review board oversight [264]. However, one could wonder on what basis individuals should be included in such trials.

1.2.3.1. Diabetes prediction

The basis for prediction of type-1 diabetes mellitus is identification of individuals with an increased genetic susceptibility to acquire type-1 diabetes (primary prediction, figure 1.1. phase I) or detection of ß-cell directed autoimmunity in individuals in the prediabetic phase (secondary prediction, figure 1.1. phase II).

1.2.3.1.1. Primary prediction

In their guidelines on genetic and immune screening for type-1 diabetes mellitus the British Diabetes Association (BDA) state "With the exception of MODY, genetic screening for identifying individuals at risk of developing diabetes is not helpful" [265]. The ADA has no guidelines on genetic screening for type-1 diabetes. However, in their recent satellite symposium report on improvement in the prognosis for type-1 diabetes, they state that screening using today's available risk markers for type-1 diabetes is ethically justified in high risk groups only, unless we can find a highly effective and safe treatment [266].

As described in paragraph 1.2.2.4.1., a strong correlation between certain HLA haplotypes and protection and susceptibility for type-1 diabetes exists. Therefore, primary prediction based on HLA susceptibility seems useful to some extent, although the results should be interpreted with caution. The applicability of HLA-typing in first degree relatives of patients with type-1 diabetes is demonstrated in table 1.3. Siblings sharing HLADR-DQ haplotypes with their proband have a 5-7 fold increased risk compared to siblings not sharing any haplotypes. Thus, concerning specific individual cases genetic counselling may be useful, but since the positive predictive value is only 20% at maximum, one may doubt if intervention based on such risk estimates is justified.

Table 1.6. demonstrates the relative risk for the general population attributed by the most important susceptibility and protective HLA-DQ haplotypes known today. There is a marked difference between different countries, which can be mainly explained by the prevalence of the alleles in the reference population, but probably also by linkage disequilibrium with other genes in the HLA-region[270]. The differences between different populations demonstrate the importance of knowing which DQ types confer diabetes risk in a particular population before the strategy for diabetes prediction is chosen.

Table 1.6. Susceptible and protective HLA-DQB1* alleles in various European populations

Susceptible	DQB1*0302 positive			DQB1*0201 positive			Ref
	T1-DM	C	RR	T1-DM	C	RR	
Belgium	34%	8%	5.5	38%	20%	2.4	(179)
Finland	73%	24%	9.0	43%	24%	2.5	(181)
Italy	35%	12%	4.1	72%	40%	3.95	(267)
Sardinia	36%	5%	11	94%	45%	19.8	(268)
Sweden	74%	26%	8.1	56%	31%	3.0	(269)
Protective	DQB1*0602 positive			DQB1*0301 positive			Ref
	T1-DM	C	RR	T1-DM	C	RR	
Belgium	0.5%	12%	0.04	6%	20%	0.24	(179)
Finland	7%	43%	0.1	n.d.	n.d.	n.d.	(181)
Italy	6%	11%	0.5	15%	48%	0.17	(267)

T1-DM: type-1 diabetes patients, C: healthy controls, RR: relative risk, Ref: reference

Table 1.6. demonstrates that just carrying one of the high risk DQB1 alleles is not sufficient to develop diabetes. Susceptible alleles can be found in up to 45% of the general population, while less than 0.5% will eventually develop type-1 diabetes. Using extended HLA-genotyping instead may improve screening but, in for example the Norwegian population, still 12.4% of the general population (accounting for 80% of the diabetes cases) carries a high risk genotype (a susceptible DQA1/DQB1 haplotype combined with any haplotype except the ones that confer protection) [27].

As described earlier the DQB1*0602 (and to a lesser extent DQB1*0201) allele is negatively associated to diabetes. Protective alleles exert their negative effect even in the presence of either DR4 or DQB1*0302. It is therefore assumed that they play a dominant protective role in the pathogenesis of type-1 diabetes (table 1.6.). Indeed relatives of patients with type-1 diabetes that are positive for ß-cell autoantibodies, but carry protective HLA-haplotypes, seem to be protected from progression to type-1 diabetes [26, 271, 272]. The presence of protective HLA DQ-haplotypes can thus be used as a negative predictor for type-1 diabetes, a strategy that may be very

powerful in combined primary and secondary prediction in both the general population and first-degree relatives.

Although other genomic regions are associated with type-1 diabetes, prediction based on these regions currently seems hardly applicable. The relative risks attributed to certain alleles are smaller and less well characterised than for HLA. However, improvement of sensitivity and specificity of primary screening may come from inclusion of additional genomic regions in genetic screening for type-1 diabetes and understanding of gene-gene interactions, once these factors have been characterised. For example, van der Auwera and colleagues demonstrate that the INS VNTR (IDDM2) has a modifying effect in subjects without the HLA-DQ genotypes associated with the highest susceptibility [25].

Thus, positive primary prediction using HLA genotyping is a sensitive but very aspecific method for diabetes prediction, a pattern that fits in the concept of diabetes being a multifactorial disease. The predictive value of screening may be improved by detection of additional risk factors (genetic risk factors, viral infections), or by the detection of the disease process itself rather than genetic susceptibility (paragraph 1.2.3.1.2).

1.2.3.1.2. Secondary prediction

Secondary prediction aims at detection of a ß-cell specific autoimmunity (figure 1.1. phase II) rather than at detection of susceptibility to the disease. Therefore, it has a higher potential specificity than primary prediction. In 90% of patients with recent onset type-1 diabetes and over 80% of individuals in the prediabetic phase ß-cell specific antibodies are detected [19, 273-277]. The appearance of these autoantibodies in the circulation is thought to reflect the autoimmune process and can therefore be used as a predictive tool for type-1 diabetes. Emerging knowledge on their target antigens and molecular biological techniques have facilitated the development of relatively

simple antibody detection systems, thus enabling the development and evaluation of secondary prediction strategies for type-1 diabetes.

1.2.3.1.2.1. Islet cell antibodies (ICA)

Before the molecular identification of ß-cell antigens, detection of ß-cell autoimmunity relied almost exclusively on the detection of islet cell antibodies (ICAs) by indirect immunofluorescence on frozen sections of human pancreas [83, 278, 279]. Due to the use of a biological substrate and the visual interpretation of the test there is large variation between different laboratories. An initiative of the Juvenile Diabetes Foundation has resulted in standardisation, using end-point titres compared to a standard serum (JDF-units). However, there is still considerable variation between different laboratories, the assay is labour intensive and quantification requires large serum volumes, thus limiting large-scale routine screening for type-1 diabetes [280-282]. Dependent on the technique used and the population studied, between 50 and 90% of patients with newly diagnosed type-1 diabetes and individuals in the prediabetic phase are positive for ICAs [279, 283-286]. The risk for progression to diabetes for first degree relatives positive for ICAs at high levels (>80JDFU) is 53 - 70% (table 1.7.) [23, 275, 287].

1.2.3.1.2.2. Insulin auto-antibodies (IAA)

Insulin autoantibodies (IAA) were the first diabetes associated antibodies recognising a molecularly defined target described [288]. From JDF standardisation workshops it has appeared that the most sensitive and specific method for their detection is a radiobindingassay (RBA) [289, 290]. The RBA requires long incubation times (up to 7 days), indicating that the antibody affinity is low. Recently, Williams and colleagues developed a micro-assay for IAA requiring smaller serum volumes and shorter incubation times. The performance of this micro-assay is comparable to the conventional RBA [291, 292]. The prevalence

of IAA is inversely correlated with age in patients, individuals in the prediabetic phase and the general population, thus limiting the use of IAA in diabetes prediction (291, 293-298). IAAs can be detected in 16-69% of patients with recent onset of type-1 diabetes (291, 295, 299, 300). The risk for progression to diabetes in first degree relatives positive for IAA is 28 -59 % by five years (table 1.7.) (275, 287, 300).

1.2.3.1.2.3. Glutamic Acid Decarboxylase (GAD)-antibodies

Other targets of autoantibodies in type-1 diabetes were detected through radio-immunoprecipitation and SDS-PAGE techniques using sera from patients with type-1 diabetes (18, 301). Since its identification in 1990, Glutamic Acid Decarboxylase (GAD) has been extensively studied, both as a target of diabetes-associated antibodies and T-cells and as a primary antigen in the pathogenesis of type-1 diabetes mellitus (18, 20, 80, 302-308). GAD is the synthesising enzyme for the inhibitory neurotransmitter gamma-amino-butyric-acid (GABA). There are two distinct isoforms of GAD, GAD_{67} and GAD_{65}, encoded by different genes. GAD_{65} and GAD_{67} are 65% identical (80% similar), the two molecules mainly differ at the N-terminal region (309-311). GAD is expressed in islets of Langerhans, neuronal tissue, ovaries and testes (312-314). In human islets of Langerhans GAD_{65} is expressed at a higher level than GAD_{67}, whereas in rat, mouse, dog and pig the contrary seems to be the case (20, 315-318). GAD_{65} is the major target the humoral immune response in human type-1 diabetes (316, 319-324).

GAD-antibodies associated with type-1 diabetes primarily recognise conformational epitopes in the middle and C-terminal region of the molecule (320, 325-331). GAD-antibody assays using isotopic (^{125}I or ^{35}S) labelled human GAD_{65} produced in combined in vitro transcription and translation techniques and precipitation of immune-complexes with protein A Sepharose have shown to be highly sensitive, specific and reproducible in JDF proficiency workshops (304, 306, 332-335). Assays

using non-isotopic methods for GAD-antibody detection are hampered by the fact that they require adsorption of GAD_{65} to plastic or labelling of GAD with relatively large groups, which may result in disruption of its native conformation [336-339].

The prevalence of GAD-antibodies varies from 60–85% in newly diagnosed patients (the lowest prevalence being reported in young children [340-343]), but GAD-antibodies can be detected in up to 3% of the general population as well, which complicates their use as a predictive marker [18, 20, 274, 295, 333, 334, 344-346]. The overall risk for GAD-antibody positive first-degree relatives to develop diabetes is 42-52% (table 1.7.) [275, 300].

1.2.3.1.2.4. Insulinoma antigen 2 (IA2) antibodies

Recently two novel receptor type proteins with homology to the protein tyrosine phosphatase family were identified and named insulinoma associated antigen 2 (IA2) and IA2ß (also known as ICA512 or phogrin) [347-349]. Before their molecular identification these antigens were described as 40 and 37 kD tryptic fragments of a 64kD autoantigen in type-1 diabetes [350]. IA2 and IA2-ß are two distinct molecules. Both are proteins with a transmembrane spanning segment and a cytoplasmatic domain that contains the epitopes for antibody recognition [351]. IA2 is expressed in peptide secreting endocrine cells and neurons containing secretory granules [351]. In patients with type-1 diabetes and prediabetic individuals antibodies are primarily directed to IA2, whereas the formation of IA2-ß antibodies is thought to be a result of epitope spreading [352].

Antibodies to IA2 are found in 48-80% of patients with newly diagnosed type-1 diabetes and prediabetes and in 2% of first-degree relatives and are correlated to rapid progression to type-1 diabetes. The overall risk for IA2 antibody positive relatives to develop diabetes within 5 years approaches 80% (table 1.7.) [275, 306, 347, 353, 354].

1.2.3.1.2.5. Other antibodies

The list of potential (ß-cell) antigens recognised by the humoral response in type-1 diabetes is constantly increasing. However, none of the other antibodies targeting ß-cell antigens recognised thus far seem to be relevant for prediction of the disease, either due to their low frequency in type-1 diabetes (carboxypeptidase H [346, 355]) or their lack of diabetes specificity (ICA69) [253]. GLIMA38 is another, yet unidentified, target antigen of the humoral response in type-1 diabetes [19, 356]. GLIMA38 shows similarities with GAD and IA2 in that it is a neuro-endocrine, membrane associated protein that is recognised via conformational epitopes. Antibodies against GLIMA38 are present in 19% of diabetic patients and 14% of prediabetic individuals up to several years prior to onset of type-1 diabetes. Until the molecular characterisation of GLIMA38 the relevance of these antibodies for diabetes prediction remains to be established.

Table 1.7. Prevalence, predictive sensitivity and positive predictive value of diabetes associated antibodies in patients with type-1 diabetes and their first degree relatives

Antibody	NDP (DS)	PS	PPV
ICA	50-90%	74-87%	53-70%
IAA	16-69%	25-76%	28-59%
GADA	60-85%	68-94%	42-52%
IA2A	48-80%	64-68%	40-80%

NDP: prevalence in newly diagnosed patients with type-1 diabetes
DS: diagnostic sensitivity
PS: predictive sensitivity in first degree relatives [275, 300]
PPV: positive predictive value in first degree relatives

1.2.3.1.2.6. Combined screening

Currently GAD and IA2 antibodies can be detected by two almost identical radiobinding assays and those assays can be even combined to produce one screening test [357]. The assays can be semi-automated (performed in 96-wells plates), use small serum volumes or even whole blood dried on filter papers, and can be easily (semi) quantified. In

addition, combined screening for GAD and IA2 antibodies can replace ICA screening [277, 346, 358, 359]. Thus, the combined analysis for GAD and IA2 antibodies represents an excellent method for the secondary prediction of type-1 diabetes.

To achieve maximum sensitivity in diabetes prediction in first-degree relatives several combinations of antibodies have been extensively tested in newly diagnosed patients with type-1 diabetes. The results of these studies are best summarised in the 1995 JDF workshop for combined antibody testing. Using two or three antibody tests screening sensitivity may be as high as 91% (mean 81%) at specificities ranging from 72-100%, depending on the threshold for positivity applied. The major drawback of these studies is that they evaluate diagnostic rather than predictive sensitivity of testing. It has been demonstrated that with longer duration of autoimmunity epitope spreading may occur [324, 352, 360-365] and studies on the validity of extrapolation of antibody results obtained in newly diagnosed diabetes to pre-diabetes are virtually lacking. Kulmala and collegues and Verge and colleagues analysed the predictive sensitivity of antibody markers in first degree relatives. Their results are summarised in table 1.7. [275, 300].

Combined screening for antibodies results in increased sensitivity and specificity and may be improved by inclusion of individuals who are positive for more than one antibody only [275, 300, 366]. Kulmala and colleagues described that of 32 siblings that progressed to diabetes during 7 years follow-up 6.3% was positive for four antibodies, 65.6% for three antibodies, 9,4% for two antibodies and 3.1 % for one antibody, while 15.6% was negative for all antibodies tested. Only 3% of siblings that did not develop diabetes were positive for multiple antibodies. Using positivity for more than one antibody as selection criterion the risk on progression to diabetes was 55%, compared to 0.9% for children who had no antibodies. Using a combination of GAD and IA2 antibodies only, the positive predictive value was 41.3%. The

sensitivity of screening was 71%, at a specificity of 95%, implicating that 40 children were false positive for these antibodies. In addition, they described that repeated screening in these sibs resulted in increased screening sensitivity since all children that progressed to diabetes did develop one or more antibodies in their follow-up sample. Thus, prediction of type-1 diabetes in first degree relatives is feasible, but may require repeated sampling. This principle is already successfully applied in the European Nicotinamide Diabetes Intervention Trial (paragraph 1.2.3.2.).

1.2.3.1.2.7. Prediction in the general population

Although the prevalence of diabetes is highest in family based populations, 90% of new cases of type-1 diabetes occur in those who have no relative with the disease. Thus, the incidence of type-1 diabetes can only be successfully decreased when it can be predicted and prevented in the general population. Simple transfer of the positive predictive value of certain prediction strategies from family members to the general population is not possible, since the prevalence of a disease largely determines the predictive value of a marker. In addition, familiar diabetes may differ from sporadic disease in pathogenesis. Thus, follow-up studies on the general population are a prerequisite. Such studies require extremely large cohorts and long follow-up since the prevalence of type-1 diabetes in the general population is low and the clinical manifestation may occur at a later age in sporadic individuals than in those who have a relative with the disease [367, 368]. Consequently, studies on prediction in the general population are rare.

The only long-term follow-up studies available today have included their cohort based on ICA positivity. One study reports similar positive predictive values for ICA positive schoolchildren compared to ICA positive sibs from patients with type-1 diabetes (40%), while in another study the positive predictive value of ICA positivity in schoolchildren was

only 6% [369, 370]. Studies are consistent in the fact that being positive for multiple antibodies is associated with a higher risk on diabetes.
Bingley and colleagues used a model comparing the distribution of antibodies in a background population to the prevalence in newly diagnosed patients to estimate the positive predictive value for several antibody combinations and screening strategies in the general population. Their model yielded risk estimates of 23% at maximum for individuals being positive for a single antibody. For children who were positive for GAD or IA2 antibodies in a first line screening and additionally positive for high levels ICA in a second antibody test the risk for progression to diabetes was 78% (65% sensitivity) [274]. However, this model again assumes that antibody patterns in prediabetes do not differ from recent onset diabetes. To evaluate if this is a valid assumption chapter 2 of this thesis evaluates the changes in the autoantibody patterns after onset of diabetes. Chapter 3 evaluates the feasibility of antibody screening for the prediction of type-1 diabetes in the general population and validity of extrapolation of data on prediction in a family based population to the general population. Based on our observations we propose a strategy for prediction of type-1 diabetes in the general population in chapter 3.1.

1.2.3.2. Diabetes prevention

Using the currentknowledge on diabetes prediction an pathogenesis two possible strategies for the prevention of type-1 diabetes can be applied:

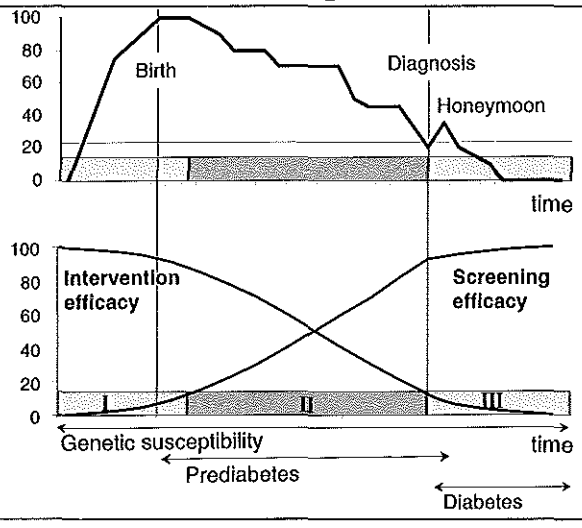
- Primary intervention: Elimination of exposures that may initiate the immune process. Potential exposures increasing the risk for type-1 diabetes have been described in paragraph 1.2.2.5. None of these factors have shown to be necessary and certainly not sufficient to initiate ß-cell autoimmunity. Therefore, it is conceivable that the attributed risk of separate exposures is low, resulting in low cost-benefit ratios for avoidance of separate risk factors. The Finnish

TRIGR (trial to reduce IDDM in the genetically at risk) aims to pilot the effect of avoidance of cow's milk on development of ß-cell autoantibodies and type-1 diabetes during the first two years of life. Children who have at least one first degree relative with type-1 diabetes and carry a HLA-DQ risk haplotype have been invited to participate in this study and recently a population based pilot has been initiated (371).

- Secondary intervention: Intervention in an ongoing autoimmune reaction resulting in prevention or delay of clinical onset of type-1 diabetes. Secondary intervention can be achieved by protection of the ß-cell (the European Nicotinamide Diabetes Intervention trial (ENDIT)) or by deviation of the immune response (Diabetes Prevention Trial (DPT)). In the ENDIT trial high risk first degree relatives are treated with high doses of nicotinamide or placebo (372, 373). Nicotinamide effectively prevents diabetes in animal models, possibly via interaction with the poly ADP-ribose (PARP) mechanism and by acting as a scavenger (374-377). In a large study among schoolchildren in New-Zealand nicotinamide treatment seems to successfully prevent or delay diabetes onset (378). The DPT trial aims to induce tolerance either by subcutaneous or oral administration of insulin to relatives at high or intermediate diabetes risk respectively (379). Both trials include first degree relatives at high risk to contract the disease based on screening for ICAs, the DPT uses additional risk staging by HLA typing, excluding those with protective HLA-phenotypes.

The effectivity of intervention strategies may largely depend on the degree of ß-cell destruction present or progression of the immune reaction (figure 1.2.). Primary intervention therefore has a higher potential effectivity. However, this requires genetic screening and, as outlined in para-graph 1.2.3.1.1., this is currently not possible without inclusion of large numbers of false positives. On the contrary, secondary

Figure 1.2: Efficacy of prediction and intervention may strongly depend on timing



intervention can be applied to individuals at high risk to progress to diabetes (identified through secondary predic-tion), but has the disadvantage that intervention may come too late to preserve enough ß-cells. The success of both strategies largely depends on our knowledge on the aetiology and pathogenesis of type-1 diabetes and our ability to identify individuals eligible to intervention. Since each intervention strategy carries the risk of dangerous side effects, the general concept of intervention must be that any pre-symptomatic treatment has a better benefit risk ratio than that of the optimal treatment of overt disease. It is important to appreciate that a large fraction of individuals participating in intervention trials will be children who can not give their own informed consent. Participation in trials means knowledge on increased risk and possibly long lasting treatment with potential hazardous drugs. In addition, we need to appreciate that with the increasing number of ongoing intervention trials the number of individuals eligible and willing to participate in new trials is rapidly decreasing. Implementation of new trials therefore requires careful consideration and international co-operation.

1.3. Aims of this thesis

Thus, in first degree relatives of patients with type-1 diabetes prediction is feasible. For relatives carrying genetic risk HLA-genotypes and being positive for more than one ß-cell antibody the risk to proceed to diabetes exceeds 60%. However, the timing to diabetes onset is still obscure and it is not clear whether data obtained in first degree relatives can be extrapolated to the general population. The aims of the studies described in this thesis were to improve diabetes prediction in first degree relatives and eventually enable extrapolation of predictive strategies to the general population. The studies in chapter 2 describe the natural course of ß-cell antibodies and their relation to disease progression, both in diabetes and prediabetes. The results provide insight in changes in autoantibody patterns occurring after diagnosis of type-1 diabetes, which is of importance to determine whether antibody data obtained in newly diagnosed patients can be extrapolated to the prediabetic phase. In addition, these studies provide knowledge on the course of antibodies in healthy first-degree relatives. These data are important for prediction purposes and may yield information on the pathophysiology of the disease. In chapter 3 the feasibility of diabetes prediction in the general population is critically evaluated and pitfalls in antibody screening are identified. Chapter 4 evaluates the technical aspects of the antibody assays that may help to solve the pitfalls identified in chapter 3.

References

1. Mandrup-Poulsen T. Diabetes. Br Med J 1998;316(7139):1221-5.
2. Leslie RDG, Taylor R, Pozilli P. The Role of Insulin Resistance in the Natural History of Type 1 Diabetes. Diabet Med 1997;14:327-331.
3. Borch-Johnsen K, Nissen H, Henriksen E, Kreiner S, Salling N, Deckert T, et al. The natural history of insulin-dependent diabetes mellitus in Denmark: 1. Long-term survival with and without late diabetic complications. Diabet Med 1987;4(3):201-10.
4. Fujimoto WY, Leonetti DL, Kinyoun JL, Shuman WP, Stolov WC, Wahl PW. Prevalence of complications among second generation Japanese-American men with diabetes, impared glucose tolerance, or normal glucose tolerance. Diabetes 1987;36:730-739.
5. Amiel SA. Diabetic control and complications. Br Med J 1993;307:881-882.
6. The Diabetes Control and Complications Trial Research Group. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. N Engl J Med 1993;329(14):977-86.
7. UK Prospective Diabetes Study (UKPDS) Group. Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34). Lancet 1998;352(9131):854-65.
8. UK Prospective Diabetes Study (UKPDS) Group. Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). Lancet 1998;352(9131):837-53.
9. Javitt JC, Chiang Y. Economic impact of diabetes. In: National Diabetes Data Group, ed Diabetes in America. Bethesda, MD: National Institutes of Health (NIH publication No 95-1168), 1995:233-255.
10. King H, Aubert RE, W.H. H. Global Burden of Diabetes, 1995-2025. Diabetes Care 1998;21(9):1414-1431.
11. UK Prospective Diabetes Study Group. Efficacy of atenolol and captopril in reducing risk of macrovascular and microvascular complications in type 2 diabetes: UKPDS 39. Brit Med J 1998;317(7160):713-20.
12. Report of the expert Committee on the Diagnosis and Classification of Diabetes Mellitus. Diabetes Care 1997;20:1183-1197.
13. Alberti KG, Zimmet PZ. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. Diabet Med 1998;15(7):539-53.
14. Harris MI, Klein R, Welborn TA, Knuiman MW. Onset of NIDDM occurs at least 4-7 years before clinical diagnosis. Diabetes Care 1992;15:815-819.
15. Mooy JM, Grootenhuis PA, de Vries H, Valkenburg HA, Bouter LM, Kostense PJ, et al. Prevalence and determinants of glucose intolerance in a Dutch caucasian population. Diabetes Care 1995;18:1270-1273.
16. Damm P, Kuhl C, Buschard K, Jakobsen BK, Svejgaard A, Sodoyez-Goffaux F, et al. Prevalence and predictive value of islet cell antibodies and insulin autoantibodies in women with gestational diabetes. Diabet Med 1994;11(6):558-63.
17. Fuchtenbusch M, Ferber K, Standl E, Ziegler AG. Prediction of type 1 diabetes postpartum in patients with gestational diabetes mellitus by combined islet cell autoantibody screening: a prospective multicenter study. Diabetes 1997;46(9):1459-67.
18. Aanstoot H.J. Identification,chracterization and application of autoantigens in type i diabetes mellitus [PhD thesis]. Erasmus University Rotterdam, 1993.

19. Aanstoot HJ, Kang SM, Kim J, Lindsay LA, Roll U, Knip M, et al. Identification and characterization of glima 38, a glycosylated islet cell membrane antigen, which together with GAD65 and IA2 marks the early phases of autoimmune response in type 1 diabetes. J Clin Invest 1996;97(12):2772-83.
20. Baekkeskov S, Aanstoot HJ, Christgau S, Reetz A, Solimena M, Cascalho M, et al. Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. Nature 1990;347(6289):151-6.
21. Seidel D, Ziegler AG. Prediction of type 1 diabetes. Horm Res 1996;45(Suppl 1):36-9.
22. Schmidli RS, Colman PG, Bonifacio E. Disease sensitivity and specificity of 52 assays for glutamic acid decarboxylase antibodies. The Second International GADAB Workshop. Diabetes 1995;44(6):636-40.
23. Bingley PJ, Christie MR, Bonifacio E, Bonfanti R, Shattock M, Fonte MT, et al. Combined analysis of autoantibodies improves prediction of IDDM in islet cell antibody-positive relatives. Diabetes 1994;43(11):1304-10.
24. Kockum I, Sanjeevi CB, Eastman S, Landinolsson M, Dahlquist G, Lernmark A, et al. Population analysis of protection by HLA-DR and DQ genes from insulin-dependent diabetes mellitus in swedish children with insulin-dependent diabetes and controls. Eur J Immunogenet 1995;22(6):443-465.
25. van der Auwera B, Schuit F, Lyaruu I, Falorni A, Svanholm S, Vandewalle CL, et al. Genetic susceptibility for insulin-dependent diabetes mellitus in Caucasians revisited: the importance of diabetes registries in disclosing interactions between HLA-DQ- and insulin gene-linked risk. Belgian Diabetes Registry. J Clin Endocrinol Metab 1995;80(9):2567-73.
26. Thorsby E, Ronningen KS. Particular HLA-DQ molecules play a dominant role in determining susceptibility or resistance to type 1 (insulin-dependent) diabetes mellitus. Diabetologia 1993;36(5):371-7.
27. Ronningen KS. Genetics in the prediction of insulin-dependent diabetes mellitus: from theory to practice. Ann Med 1997;29(5):387-92.
28. Ruwaard D, Hirasing RA, Reeser HM, van Buuren S, Bakker K, Heine RJ, et al. Increasing incidence of type I diabetes in The Netherlands. The second nationwide study among children under 20 years of age. Diabetes Care 1994;17(6):599-601.
29. Reeser HM. Epidemiology of childhood diabetes in The Netherlands [PhD-thesis]. Leiden State University, 1998.
30. Ruwaard D, Gijsen R, Bartelds AI, Hirasing RA, Verkleij H, Kromhout D. Is the incidence of diabetes increasing in all age-groups in The Netherlands? Results of the second study in the Dutch Sentinel Practice Network. Diabetes Care 1996;19(3):214-8.
31. Dahlquist G, Mustonen L. Childhood onset diabetes--time trends and climatological factors. Med J Aust 1995;162(2):111.
32. Gardner SG, Bingley PJ, Sawtell PA, Weeks S, Gale EA. Rising incidence of insulin dependent diabetes in children aged under 5 years in the Oxford region: time trend analysis. The Bart's-Oxford Study Group. Brit Med J 1997;315(7110):713-7.
33. Karvonen M, Tuomilehto J, Libman I, LaPorte R. A review of the recent epidemiological data on the worldwide incidence of type 1 (insulin-dependent) diabetes mellitus. World Health Organization DIAMOND Project Group. Diabetologia 1993;36(10):883-92.
34. Tuomilehto J, Rewers M, Reunanen A, Lounamaa P, Lounamaa R, Tuomilehto-Wolf E, et al. Increasing trend in type 1 (insulin-dependent) diabetes mellitus in childhood in Finland. Analysis of age, calendar time and birth cohort effects during 1965 to 1984. Diabetologia 1991;34(4):282-7.
35. Bingley PJ, Gale EA. Rising incidence of IDDM in Europe. Diabetes Care 1989;12(4):289-95.

36. Christau B, Kromann H, Andersen OO, Christy M, Buschard K, Arnung K, et al. Incidence, seasonal and geographical patterns of juvenile-onset insulin-dependent diabetes mellitus in Denmark. Diabetologia 1977;13(4):281-4.
37. Dahlquist GG, Kallen BA. Time-space clustering of date at birth in childhood-onset diabetes. Diabetes Care 1996;19(9):1035-6.
38. Karvonen M, Jantti V, Muntoni S, Stabilini M, Stabilini L, Tuomilehto J. Comparison of the seasonal pattern in the clinical onset of IDDM in Finland and Sardinia. Diabetes Care 1998;21(7):1101-9.
39. Nystrom L, Dahlquist G, Ostman J, Wall S, Arnqvist H, Blohme G, et al. Risk of developing insulin-dependent diabetes mellitus (IDDM) before 35 years of age: indications of climatological determinants for age at onset. Diabet Med 1992;9(3):279-84.
40. Bosi E, Sarugeri E. Advances and controversies in etiopathogenesis of type 1 (insulin- dependent) diabetes mellitus. J Pediatr Endocrinol Metab 1998;11(Suppl 2):293-305.
41. Dahlquist GG, Ivarsson S, Lindberg B, Fosgren M. Maternal enteroviral infection during pregnancy as a risk factor for childhood IDDM. Diabetes 1995;44:408-413.
42. Rothwell PM, Staines A, Smail P, Wadsworth E, McKinney P. Seasonality of birth of patients with childhood diabetes in Britain. Brit Med J 1996;312(7044):1456-7.
43. Jongbloet PH, Groenewoud HM, Hirasing RA, Van Buuren S. Seasonality of birth in patients with childhood diabetes in The Netherlands. Diabetes Care 1998;21(1):190-1.
44. Rothwell PM. Interpretation of temporal variation in patterns of birth. Lancet 1994;344(8930):1161-2.
45. Helgason T, Ewen SW, Jaffray B, Stowers JM, Outram JR, Pollock JR. N-nitrosamines in smoked meats and their relation to diabetes. IARC Sci Publ 1984;57:911-20.
46. Diabetes Epidemiology Research International Group. Secular trends in incidence of childhood IDDM in 10 countries. Ann Med 1991;23(4):415-7.
47. Tuomilehto J, Lounamaa R, Tuomilehto-Wolf E, Reunanen A, Virtala E, Kaprio EA, et al. Epidemiology of childhood diabetes mellitus in Finland--background of a nationwide study of type 1 (insulin-dependent) diabetes mellitus. The Childhood Diabetes in Finland (DiMe) Study Group. Diabetologia 1992;35(1):70-6.
48. Green A, Gale EA, Patterson CC. Incidence of childhood onset insulin dependent diabetes mellitus:the EURODIAB ACE Study. Lancet 1992;339(8798):905-909.
49. Muntoni S, Stabilini L, Stabilini M, Mancosu G, Muntoni S. Steadily high IDDM incidence over 4 years in sardinia. Diabetes Care 1995;18(12):1600-1601.
50. Muntoni S, Fonte MT, Stoduto S, Marietti G, Bizzarri C, Crino A, et al. Incidence of insulin-dependent diabetes mellitus among Sardinian- heritage children born in Lazio region, Italy. Lancet 1997;349(9046):160-2.
51. Bingley PJ, Gale EA. Genetics of diabetes. Lessons from family studies. Baillieres Clin Endocrinol Metab 1991;5(2):261-83.
52. Irvine WJ, Sawers JS, Feek CM, Prescott RJ, Duncan LJ. The value of islet cell antibody in predicting secondary failure of oral hypoglycaemic agent therapy in Diabetes mellitus. J Clin Lab Immunol 1979;2(1):23-6.
53. Niskanen LK, Tuomi T, Karjalainen J, Groop LC, Uusitupa MIJ. GAD antibodies in NIDDM: ten-year follow-up from the diagnosis. Diabetes Care 1995;18(12):1557-1565.
54. Rowley MJ, Mackay IR, Chen QY, Knowles WJ, Zimmet PZ. Antibodies to glutamic acid decarboxylase discriminate major types of diabetes mellitus. Diabetes 1992;41(4):548-51.
55. Kobayashi T, Nakanishi K, Okubo M, Murase T, Kosaka K. GAD antibodies seldom disappear in slowly progressive IDDM. Diabetes Care 1996;19(9):1031.

56. Gottsater A, Landin-Olsson M, Fernlund P, Lernmark A, Sundkvist G. Beta-cell function in relation to islet cell antibodies during the first 3 yr after clinical diagnosis of diabetes in type II diabetic patients. Diabetes Care 1993;16(6):902-10.
57. Groop LC, Bottazzo GF, Doniach D. Islet cell antibodies identify latent type I diabetes in patients aged 35-75 years at diagnosis. Diabetes 1986;35(2):237-41.
58. Turner R, Stratton I, Horton V, Manley S, Zimmet P, Mackay IR, et al. UKPDS 25: autoantibodies to islet-cell cytoplasm and glutamic acid decarboxylase for prediction of insulin requirement in type 2 diabetes. UK Prospective Diabetes Study Group. Lancet 1997;350(9087):1288-93.
59. Zimmet PZ. The pathogenesis and prevention of diabetes in adults: genes, autoimmunity, and demography. Diabetes Care 1995;18(7):1050-1064.
60. Zimmet PZ, Tuomi T, Mackay IR, Rowley MJ, Knowles W, Cohen M, et al. Latent autoimmune diabetes mellitus in adults (LADA): the role of antibodies to glutamic acid decarboxylase in diagnosis and prediction of insulin dependency. Diabet Med 1994;11(3):299-303.
61. Vandewalle CL, Decraene T, Schuit FC, De Leeuw IH, Pipeleers DG, Gorus FK. Insulin autoantibodies and high titre islet cell antibodies are preferentially associated with the HLA DQA1*0301-DQB1*0302 haplotype at clinical type 1 (insulin-dependent) diabetes mellitus before age 10 years, but not at onset between age 10 and 40 years. The Belgian Diabetes Registry. Diabetologia 1993;36(11):1155-62.
62. Rahier J, Goebbels RM, Henquin JC. Cellular composition of the human diabetic pancreas. Diabetologia 1983;24(5):366-71.
63. Waguri M, Hanafusa T, Itoh N, Miyagawa J, Imagawa A, Kuwajima M, et al. Histopathologic study of the pancreas shows a characteristic lymphocytic infiltration in Japanese patients with IDDM. Endocr J 1997;44(1):23-33.
64. Foulis AK, Farquharson MA, Hardman R. Aberrant expression of class II major histocompatibility complex molecules by B cells and hyperexpression of class I major histocompatibility complex molecules by insulin containing islets in type 1 (insulin-dependent) diabetes mellitus. Diabetologia 1987;30(5):333-43.
65. Gepts W. Pathologic anatomy of the pancreas in juvenile diabetes mellitus. Diabetes 1965;14:619-633.
66. Bottazzo GF, Dean BM, McNally JM, MacKay EH, Swift PG, Gamble DR. In situ characterization of autoimmune phenomena and expression of HLA molecules in the pancreas in diabetic insulitis. N Engl J Med 1985;313(6):353-60.
67. Foulis AK, Stewart JA. The pancreas in recent-onset type 1 (insulin-dependent) diabetes mellitus: insulin content of islets, insulitis and associated changes in the exocrine acinar tissue. Diabetologia 1984;26(6):456-61.
68. Foulis AK, Liddle CN, Farquharson MA, Richmond JA, Weir RS. The histopathology of the pancreas in type 1 (insulin-dependent) diabetes mellitus: a 25-year review of deaths in patients under 20 years of age in the United Kingdom. Diabetologia 1986;29(5):267-74.
69. Itoh N, Hanafusa T, Miyazaki A, Miyagawa J, Yamagata K, Yamamoto K, et al. Mononuclear cell infiltration and its relation to the expression of major histocompatibility complex antigens and adhesion molecules in pancreas biopsy specimens from newly diagnosed insulin-dependent diabetes mellitus patients. J Clin Invest 1993;92(5):2313-22.
70. Conrad B, Weidmann E, Trucco G, Rudert WA, Behboo R, Ricordi C, et al. Evidence for superantigen involvement in insulin-dependent diabetes mellitus aetiology. Nature 1994;371(6495):351-5.
71. Lernmark A, Kloppel G, Stenger D, Vathanaprida C, Falt K, Landin-Olsson M, et al. Heterogeneity of islet pathology in two infants with recent onset diabetes mellitus. Virchows Arch 1995;425(6):631-40.

72. Makino S, Kunimoto K, Muraoka Y, Mizushima Y, Katagiri K, Tochino Y. Breeding of a non-obese diabetic strain of mice. Exp Anim 1980;29:1-3.
73. Jansen A, Homo-Delarche F, Hooijkaas H, Leenen PJ, Dardenne M, Drexhage HA. Immunohistochemical characterization of monocytes-macrophages and dendritic cells involved in the initiation of the insulitis and beta- cell destruction in NOD mice. Diabetes 1994;43(5):667-75.
74. Shimada A, Charlton B, Taylor-Edwards C, Fathman CG. Beta-cell destruction may be a late consequence of the autoimmune process in nonobese diabetic mice. Diabetes 1996;45(8):1063-7.
75. von Herrath M, Holz A. Pathological changes in the islet milieu precede infiltration of islets and destruction of beta-cells by autoreactive lymphocytes in a transgenic model of virus-induced IDDM. J Autoimmun 1997;10(3):231-8.
76. Holers VM, Hollis GF, Schwartz BD, Evans RJ, Strauss-Schoenberger J, Carel JC, et al. Induction of peri-insulitis but not diabetes in islet transplants expressing a single foreign antigen. A multi-stage model of disease. J Immunol 1993;151(9):5041-52.
77. Reddy S, Liu W, Thompson JM, Bibby NJ, Elliott RB. First phase insulin release in the non-obese diabetic mouse: correlation with insulitis, beta cell number and autoantibodies. Diabetes Res Clin Pract 1992;17(1):17-25.
78. Andre I, Gonzalez A, Wang B, Katz J, Benoist C, Mathis D. Checkpoints in the progression of autoimmune disease: lessons from diabetes models. Proc Natl Acad Sci U S A 1996;93(6):2260-3.
79. Tarn AC, Smith CP, Spencer KM, Bottazzo GF, Gale EA. Type I (insulin dependent) diabetes: a disease of slow clinical onset? Br Med J 1987;294(6568):342-5.
80. Aanstoot HJ, Sigurdsson E, Jaffe M, Shi Y, Christgau S, Grobbee D, et al. Value of antibodies to GAD65 combined with islet cell cytoplasmic antibodies for predicting IDDM in a childhood population. Diabetologia 1994;37(9):917-24.
81. Baekkeskov S, Dyrberg T, Lernmark A. Autoantibodies to a 64-kilodalton islet cell protein precede the onset of spontaneous diabetes in the BB rat. Science 1984;224(4655):1348-50.
82. Baekkeskov S, Landin M, Kristensen JK, Srikanta S, Bruining GJ, Mandrup-Poulsen T, et al. Antibodies to a 64,000 Mr human islet cell antigen precede the clinical onset of insulin-dependent diabetes. J Clin Invest 1987;79(3):926-34.
83. Bingley PJ, Bonifacio E, Gale EA. Can we really predict IDDM? Diabetes 1993;42(2):213-20.
84. Bosi E, Bonifacio E. Autoantibodies in insulin-dependent diabetes mellitus. J Endocrinol invest 1994;17(7):521-31.
85. Bruining GJ, Molenaar JL, Grobbee DE, Hofman A, Scheffer GJ, Bruining HA, et al. Ten-year follow-up study of islet-cell antibodies and childhood diabetes mellitus. Lancet 1989;1(8647):1100-3.
86. De Aizpurua HJ, Wilson YM, Harrison LC. Glutamic acid decarboxylase autoantibodies in preclinical insulin- dependent diabetes. Proc Natl Acad Sci U S A 1992;89(20):9841-5.
87. Movassat J, Saulnier C, Portha B. Insulin administration enhances growth of the beta-cell mass in streptozotocin-treated newborn rats. Diabetes 1997;46(9):1445-52.
88. Thivolet C, Bendelac A, Bedossa P, Bach JF, Carnaud C. CD8+ T cell homing to the pancreas in the nonobese diabetic mouse is CD4+ T cell-dependent. J Immunol 1991;146(1):85-8.
89. Wicker LS, Todd JA, Peterson LB. Genetic control of autoimmune diabetes in the NOD mouse. Annu Rev Immunol 1995;13:179-200.
90. Healey D, Ozegbe P, Arden S, Chandler P, Hutton J, Cooke A. In vivo activity and in vitro specificity of CD4+ Th1 and Th2 cells derived from the spleens of diabetic NOD mice. J Clin Invest 1995;95(6):2979-85.

91. Romagnani S. The Th1/Th2 paradigm. Immunol Today 1997;18(6):263-6.
92. Katz JD, Benoist C, Mathis D. T helper cell subsets in insulin-dependent diabetes. Science 1995;268(5214):1185-8.
93. Akhtar I, Gold JP, Pan LY, Ferrara JL, Yang XD, Kim JI, et al. CD4+ beta islet cell-reactive T cell clones that suppress autoimmune diabetes in nonobese diabetic mice. J Exp Med 1995;182(1):87-97.
94. Trembleau S, Penna G, Gregori S, Gately MK, Adorini L. Deviation of pancreas-infiltrating cells to Th2 by interleukin-12 antagonist administration inhibits autoimmune diabetes. Eur J Immunol 1997;27(9):2330-9.
95. Sallusto F, Lanzavecchia A, Mackay CR. Chemokines and chemokine receptors in T-cell priming and Th1/Th2- mediated responses. Immunol Today 1998;19(12):568-74.
96. Romagnani S. Induction of TH1 and TH2 responses: a key role for the 'natural' immune response? Immunol Today 1992;13(10):379-81.
97. Abbas AK, Murphy KM, Sher A. Functional diversity of helper T lymphocytes. Nature 1996;383(6603):787-93.
98. Tisch R, McDevitt H. Insulin-dependent diabetes mellitus. Cell 1996;85(3):291-7.
99. Piganelli JD, Martin T, Haskins K. Splenic macrophages from the NOD mouse are defective in the ability to present antigen. Diabetes 1998;47(8):1212-8.
100. Jansen A, van Hagen M, Drexhage HA. Defective maturation and function of antigen-presenting cells in type 1 diabetes. Lancet 1995;345(8948):491-2.
101. Rothe H, Kolb H. The APC1 concept of type I diabetes. Autoimmunity 1998;27(3):179-84.
102. de St Groth BF. The evolution of self-tolerance: a new cell arises to meet the challenge of self-reactivity. Immunol Today 1998;19(10):448-54.
103. Jun HS, Yoon CS, Zbytnuik L, van Rooijen N, Yoon JW. The role of macrophages in T cell-mediated autoimmune diabetes in nonobese diabetic mice. J Exp Med 1999;189(2):347-58.
104. Simons PJ, Delemarre FG, Jeucken PH, Drexhage HA. Pre-autoimmune thyroid abnormalities in the biobreeding diabetes-prone (BB-DP) rat: a possible relation with the intrathyroid accumulation of dendritic cells and the initiation of the thyroid autoimmune response. J Endocrinol 1998;157(1):43-51.
105. Allaerts W, Tijssen AM, Jeucken PH, Drexhage HA, de Koning J. Influence of folliculo-stellate cells on biphasic luteinizing hormone secretion response to gonadotropin-releasing hormone in rat pituitary cell aggregates. Eur J Endocrinol 1994;130(5):530-9.
106. Hoek A, Allaerts W, Leenen PJ, Schoemaker J, Drexhage HA. Dendritic cells and macrophages in the pituitary and the gonads. Evidence for their role in the fine regulation of the reproductive endocrine response. Eur J Endocrinol 1997;136(1):8-24.
107. Jansen A, Voorbij HA, Jeucken PH, Bruining GJ, Hooijkaas H, Drexhage HA. An immunohistochemical study on organized lymphoid cell infiltrates in fetal and neonatal pancreases. A comparison with similar infiltrates found in the pancreas of a diabetic infant. Autoimmunity 1993;15(1):31-8.
108. Martin S, Heidenthal E, Schulte B, Rothe H, Kolb H. Soluble forms of intercellular adhesion molecule-1 inhibit insulitis and onset of autoimmune diabetes. Diabetologia 1998;41(11):1298-303.
109. Andre-Schmutz I, Hindelang C, Benoist C, Mathis D. Cellular and molecular changes accompanying the progression from insulitis to diabetes. Eur J Immunol 1999;29(1):245-55.
110. Goodnow CC. Balancing immunity and tolerance: deleting and tuning lymphocyte repertoires. Proc Natl Acad Sci USA 1996;93(6):2264-2271.
111. Rabinovitch A, Skyler JS. Prevention of type 1 diabetes. Med Clin North Am 1998;82(4):739-55.

112. Boitard C, Timsit J, Larger E, Sempe P, Bach JF. Pathogenesis of IDDM: immune regulation and induction of immune tolerance in the NOD mouse. Autoimmunity 1993;15:12-3.
113. Bach JF. Strategies in immunotherapy of insulin-dependent diabetes mellitus. Ann N Y Acad Sci 1993;696:364-76.
114. Aichele P, Brduschariem K, Zinkernagel RM, Hengartner H, Pircher H. T cell priming versus t cell tolerance induced by synthetic peptides. Journal of Experimental Medicine 1995;182(1):261-266.
115. Kolb H, Worz-Pagenstert U, Kleemann R, Rothe H, Rowsell P, Rastegar S, et al. Insulin therapy of prediabetes suppresses TH1 associated gene expression in BB rat pancreas. Autoimmunity 1997;26(1):1-6.
116. Falcone M, Lee J, Patstone G, Yeung B, Sarvetnick N. B lymphocytes are crucial antigen-presenting cells in the pathogenic autoimmune response to GAD65 antigen in nonobese diabetic mice. J Immunol 1998;161(3):1163-8.
117. Kelso A. Th1 and Th2 subsets: paradigms lost? Immunol Today 1995;16(8):374-9.
118. Mosmann TR, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. Immunol Today 1996;17(3):138-46.
119. Muraille E, Leo O. Revisiting the Th1/Th2 paradigm. Scand J Immunol 1998;47(1):1-9.
120. Heurtier AH, Boitard C. T-cell regulation in murine and human autoimmune diabetes: the role of TH1 and TH2 cells. Diabetes Metab 1997;23(5):377-85.
121. Del Prete G, De Carli M, Almerigogna F, Giudizi MG, Biagiotti R, Romagnani S. Human IL-10 is produced by both type 1 helper (Th1) and type 2 helper (Th2) T cell clones and inhibits their antigen-specific proliferation and cytokine production. J Immunol 1993;150(2):353-60.
122. Romagnani S. Th1 and Th2 in human diseases. Clin Immunol Immunopathol 1996;80(3 Pt 1):225-35.
123. Romagnani S. Biology of human TH1 and TH2 cells. J Clin Immunol 1995;15(3):121-9.
124. Zhu J, Mix E, Link H. Cytokine production and the pathogenesis of experimental autoimmune neuritis and Guillain-Barre syndrome. J Neuroimmunol 1998;84(1):40-52.
125. Roep BO. T-cell responses to autoantigens in IDDM. The search for the Holy Grail. Diabetes 1996;45(9):1147-56.
126. Schloot NC, Roep BO, Wegmann D, Yu L, Chase HP, Wang T, et al. Altered immune response to insulin in newly diagnosed compared to insulin-treated diabetic patients and healthy control subjects. Diabetologia 1997;40(5):564-72.
127. Al-Sakkaf L, Pozzilli P, Tarn AC, Schwarz G, Gale EA, Bottazzo GF. Persistent reduction of CD4/CD8 lymphocyte ratio and cell activation before the onset of type 1 (insulin-dependent) diabetes. Diabetologia 1989;32(5):322-5.
128. Scheinin T, Maenpaa J, Koskimies S, Dean BM, Bottazzo GF, Kontiainen S. Insulin responses and lymphocyte subclasses in children with newly diagnosed insulin-dependent diabetes. Clin Exp Immunol 1988;71(1):91-5.
129. Harrison LC, De Aizpurua H, Loudovaris T, Campbell IL, Cebon JS, Tait BD, et al. Reactivity to human islets and fetal pig proislets by peripheral blood mononuclear cells from subjects with preclinical and clinical insulin- dependent diabetes. Diabetes 1991;40(9):1128-33.
130. Roep BO, Kallan AA, Duinkerken G, Arden SD, Hutton JC, Bruining GJ, et al. T-cell reactivity to beta-cell membrane antigens associated with beta- cell destruction in IDDM. Diabetes 1995;44(3):278-83.
131. Roep BO, Arden SD, de Vries RR, Hutton JC. T-cell clones from a type-1 diabetes patient respond to insulin secretory granule proteins. Nature 1990;345(6276):632-4.

132. Roep BO, Kallan AA, Hazenbos WL, Bruining GJ, Bailyes EM, Arden SD, et al. T-cell reactivity to 38 kD insulin-secretory-granule protein in patients with recent-onset type 1 diabetes. Lancet 1991;337(8755):1439-41.
133. Atkinson MA, Kaufman DL, Campbell L, Gibbs KA, Shah SC, Bu DF, et al. Response of peripheral-blood mononuclear cells to glutamate decarboxylase in insulin-dependent diabetes. Lancet 1992;339(8791):458-9.
134. Honeyman MC, Cram DS, Harrison LC. Transcription factor jun-B is target of autoreactive T-cells in IDDM. Diabetes 1993;42(4):626-30.
135. Bonifacio E, Boitard C. T-cell reactivity to 38 kD insulin-secretory granule protein in patients with recent onset type 1 diabetes. J Endocrinol Invest 1994;17(7):559-63.
136. Arden SD, Roep BO, Neophytou PI, Usac EF, Duinkerken G, de Vries RR, et al. Imogen 38: a novel 38-kD islet mitochondrial autoantigen recognized by T cells from a newly diagnosed type 1 diabetic patient. J Clin Invest 1996;97(2):551-61.
137. Schloot NC, Willemen S, Duinkerken G, de Vries RR, Roep BO. Cloned T cells from a recent onset IDDM patient reactive with insulin B- chain. J Autoimmun 1998;11(2):169-75.
138. Lohmann T, Scherbaum WA. T cell autoimmunity to glutamic acid decarboxylase in human insulin-dependent diabetes mellitus. Horm Metab Res 1996;28(7):357-360.
139. Lohmann T, Leslie R, Londei M. T cell clones to epitopes of glutamic acid decarboxylase 65 raised from normal subjects and patients with insulin-dependent diabetes. J Autoimmun 1996;9(3):385-389.
140. Atkinson MA, Bowman MA, Campbell L, Darrow BL, Kaufman DL, Maclaren NK. Cellular immunity to a determinant common to glutamate decarboxylase and coxsackie virus in insulin-dependent diabetes. J Clin Invest 1994;94(5):2125-9.
141. Vreugdenhil GR, Geluk A, Ottenhoff TH, Melchers WJ, Roep BO, Galama JM. Molecular mimicry in diabetes mellitus: the homologous domain in coxsackie B virus protein 2C and islet autoantigen GAD65 is highly conserved in the coxsackie B-like enteroviruses and binds to the diabetes associated HLA-DR3 molecule. Diabetologia 1998;41(1):40-6.
142. Hou J, Said C, Franchi D, Dockstader P, Chatterjee NK. Antibodies to glutamic acid decarboxylase and P2-C peptides in sera from coxsackie virus B4-infected mice and IDDM patients. Diabetes 1994;43(10):1260-6.
143. Durinovic-Bello I. Autoimmune diabetes: the role of T cells, MHC molecules and autoantigens. Autoimmunity 1998;27(3):159-77.
144. Durinovic-Bello I, Hummel M, Ziegler AG. Cellular immune response to diverse islet cell antigens in IDDM. Diabetes 1996;45(6):795-800.
145. Ellis TM, Schatz DA, Ottendorfer EW, Lan MS, Wasserfall C, Salisbury PJ, et al. The relationship between humoral and cellular immunity to IA-2 in IDDM. Diabetes 1998;47(4):566-9.
146. Honeyman MC, Stone NL, Harrison LC. T-cell epitopes in type 1 diabetes autoantigen tyrosine phosphatase IA- 2: potential for mimicry with rotavirus and other environmental agents. Mol Med 1998;4(4):231-9.
147. Homo-Delarche F. ß-cell behaviour during the pre-diabetic stage. Diabetes & Metabolism 1997;23:181-194.
148. Homo-Delarche F, Boitard C. Autoimmune diabetes: the role of the islets of Langerhans. Immunol Today 1996;17(10):456-60.
149. Kaprio J, Tuomilehto J, Koskenvuo M, Romanov K, Reunanen A, Eriksson J, et al. Concordance for type 1 (insulin-dependent) and type 2 (non-insulin-dependent) diabetes mellitus in a population-based cohort of twins in Finland. Diabetologia 1992;35(11):1060-7.
150. Kumar D, Gemayel NS, Deapen D, Kapadia D, Yamashita PH, Lee M, et al. North-American twins with IDDM. Genetic, etiological, and clinical significance

of disease concordance according to age, zygosity, and the interval after diagnosis in first twin. Diabetes 1993;42(9):1351-63.
151. The Eurodiab Ace Study Group and The Eurodiab Ace Substudy 2 Study Group. Familial risk of type I diabetes in European children. Diabetologia 1998;41(10):1151-6.
152. Thomson G, Robinson WP, Kuhner MK, Joe S, MacDonald MJ, Gottschall JL, et al. Genetic heterogeneity, modes of inheritance, and risk estimates for a joint study of Caucasians with insulin-dependent diabetes mellitus. Am J Hum Genet 1988;43(6):799-816.
153. Hawkes CH. Twin studies in diabetes mellitus. Diabet Med 1997;14(5):347-52.
154. Leslie RDG, Pyke DA. Concordance between twins for beta-cell autoimmunity. Diabetes 1996;45(9):1284.
155. Kyvik KO, Green A, Beck-Nielsen H. The new Danish Twin Register: establishment and analysis of twinning rates. J Clin Endocrinol Metab 1995;80(9):2567-73.
156. Petersen JS, Kyvik KO, Bingley PJ, Gale EA, Green A, Dyrberg T, et al. Population based study of prevalence of islet cell autoantibodies in monozygotic and dizygotic Danish twin pairs with insulin dependent diabetes mellitus. Brit Med J 1997;314(7094):1575-9.
157. Todd JA, Bell JI, McDevitt HO. HLA-DQ beta gene contributes to susceptibility and resistance to insulin-dependent diabetes mellitus. Nature 1987;329(6140):599-604.
158. Davies JL, Kawaguchi Y, Bennett ST, Copeman JB, Cordell HJ, Pritchard LE, et al. A genome-wide search for human type 1 diabetes susceptibility genes. Nature 1994;371(6493):130-6.
159. Rotter JI, Landaw EM. Measuring the genetic contribution of a single locus to a multilocus disease. Clin Genet 1984;26(6):529-42.
160. Nerup J, PLatz P, Andersen OO, Christy M, Lyngsoe J, Poulsen JE, et al. HLA antigens and diabetes mellitus. Lancet 1974;2:864-866.
161. Khalil I, d'Auriol L, Gobet M, Morin L, Lepage V, Deschamps I, et al. A combination of HLA-DQ beta Asp57-negative and HLA DQ alpha Arg52 confers susceptibility to insulin-dependent diabetes mellitus. HLA DQ alpha/beta molecule associated with the susceptibility to insulin-dependent diabetes mellitus. A model for HLA/autoimmune disease association. J Clin Invest 1990;85(4):1315-9.
162. Baisch JM, Weeks T, Giles R, Hoover M, Stastny P, Capra JD. Analysis of HLA-DQ genotypes and susceptibility in insulin-dependent diabetes mellitus. N Engl J Med 1990;322(26):1836-41.
163. Giphart MJ, Roep BO, Drabbels J, D'Amaro J, Bruining GJ, Abdulkadir J, et al. Relative contribution of HLA-DQA and -DQB alleles to predisposition to insulin-dependent diabetes mellitus. Hum Immunol 1992;34(2):142-6.
164. Morel PA, Dorman JS, Todd JA, McDevitt HO, Trucco M. Aspartic acid at position 57 of the HLA-DQ beta chain protects against type I diabetes: a family study Proc Natl Acad Sci U S A 1988;85(21):8111-5.
165. Zamani Ghabanbasani M, Spaepen M, Buyse I, Marynen P, Bex M, Bouillon R, et al. Improved risk assessment for insulin-dependent diabetes mellitus by analysis of amino acids in HLA-DQ and DRB1 loci. Eur J Hum Genet 1994;2(3):177-84.
166. Zamani M, Cassiman JJ. Reevaluation of the importance of polymorphic HLA class II alleles and amino acids in the susceptibility of individuals of different populations to type I diabetes. Am J Med Genet 1998;76(2):183-94.
167. Slattery R. Transgenic approaches to understanding the role of MHC genes in insulin dependent diabetes mellitus. II. The non-obese diabetic (NOD) mouse. Baillieres Clin Endocrinol Metab 1991;5(3):449-54.

168. Slattery RM, Kjer-Nielsen L, Allison J, Charlton B, Mandel TE, Miller JF. Prevention of diabetes in non-obese diabetic I-Ak transgenic mice. Nature 1990;345(6277):724-6.
169. Cornall RJ, Prins JB, Todd JA, Pressey A, DeLarato NH, Wicker LS, et al. Type 1 diabetes in mice is linked to the interleukin-1 receptor and Lsh/Ity/Bcg genes on chromosome 1. Nature 1991;353(6341):262-5.
170. Copeman JB, Cucca F, Hearne CM, Cornall RJ, Reed PW, Ronningen KS, et al. Linkage disequilibrium mapping of a type 1 diabetes susceptibility gene (IDDM7) to chromosome 2q31-q33. Nat Genet 1995;9(1):80-5.
171. Nistico L, Buzzetti R, Pritchard LE, Vanderauwera B, Giovannini C, Bosi E, et al. The CTLA-4 gene region of chromosome 2q33 is linked to, and associated with, type 1 diabetes. Hum Mol Genet 1996;5(7):1075-1080.
172. Todd JA, Farrall M. Panning for gold: genome-wide scanning for linkage in type 1 diabetes. Hum Mol Genet 1996;5(Spec No):1443-8.
173. Mein CA, Esposito L, Dunn MG, Johnson GC, Timms AE, Goy JV, et al. A search for type 1 diabetes susceptibility genes in families from the United Kingdom. Nat Genet 1998;19(3):297-300.
174. Concannon P, Gogolin-Ewens KJ, Hinds DA, Wapelhorst B, Morrison VA, Stirling B, et al. A second-generation screen of the human genome for susceptibility to insulin-dependent diabetes mellitus. Nat Genet 1998;19(3):292-6.
175. Lernmark A, Ott J. Sometimes it's hot, sometimes it's not. Nat Genet 1998;19(3):213-4.
176. Cordell HJ, Todd JA. Multifactorial inheritance in type 1 diabetes. Trends Genet 1995;11(12):499-504.
177. Reich EP, von Grafenstein H, Barlow A, Swenson KE, Williams K, Janeway CA, Jr. Self peptides isolated from MHC glycoproteins of non-obese diabetic mice. J Immunol 1994;152(5):2279-88.
178. Rammensee HG. Chemistry of peptides associated with MHC class I and class II molecules. Curr Opin Immunol 1995;7(1):85-96.
179. Buyse I, Sandkuyl LA, Zamani Ghabanbasani M, Gu XX, Bouillon R, Bex M, et al. Association of particular HLA class II alleles, haplotypes and genotypes with susceptibility to IDDM in the Belgian population. Diabetologia 1994;37(8):808-17.
180. Ilonen J, Surcel HM, Partanen J, Koskimies S, Knip M, Kaar ML. Extended HLA haplotypes in families with insulin-dependent diabetes mellitus in northern Finland. Tissue Antigens 1988;32(3):139-44.
181. Ilonen J, Reijonen H, Herva E, Sjoroos M, Iitia A, Lovgren T, et al. Rapid HLA-DQB1 genotyping for four alleles in the assessment of risk for IDDM in the Finnish population. The Childhood Diabetes in Finland (DiMe) Study Group. Diabetes Care 1996;19(8):795-800.
182. Bell GI, Horita S, Karam JH. A polymorphic locus near the human insulin gene is associated with insulin-dependent diabetes mellitus. Diabetes 1984;33(2):176-83.
183. Bennett ST, Lucassen AM, Gough SC, Powell EE, Undlien DE, Pritchard LE, et al. Susceptibility to human type 1 diabetes at IDDM2 is determined by tandem repeat variation at the insulin gene minisatellite locus. Nat Genet 1995;9(3):284-92.
184. Bennett ST, Wilson AJ, Cucca F, Nerup J, Pociot F, McKinney PA, et al. IDDM2-VNTR-encoded susceptibility to type 1 diabetes: dominant protection and parental transmission of alleles of the insulin gene- linked minisatellite locus. J Autoimmun 1996;9(3):415-21.
185. Kennedy GC, German MS, Rutter WJ. The minisatellite in the diabetes susceptibility locus IDDM2 regulates insulin transcription. Nat Genet 1995;9(3):293-8.
186. Pugliese A, Zeller M, Fernandez A, Jr., Zalcberg LJ, Bartlett RJ, Ricordi C, et al. The insulin gene is transcribed in the human thymus and transcription levels

correlated with allelic variation at the INS VNTR-IDDM2 susceptibility locus for type 1 diabetes. Nat Genet 1997;15(3):293-7.
187. Vafiadis P, Bennett ST, Todd JA, Nadeau J, Grabs R, Goodyer CG, et al. Insulin expression in human thymus is modulated by INS VNTR alleles at the IDDM2 locus. Nat Genet 1997;15(3):289-92.
188. Sarugeri E, Dozio N, Belloni C, Meschi F, Pastore MR, Bonifacio E. Autoimmune responses to the beta cell autoantigen, insulin, and the INS VNTR-IDDM2 locus. Clin Exp Immunol 1998;114(3):370-6.
189. Awata T, Kurihara S, Iitaka M, Takei S, Inoue I, Ishii C, et al. Association of CTLA-4 gene A-G polymorphism (IDDM12 locus) with acute- onset and insulin-depleted IDDM as well as autoimmune thyroid disease (Graves' disease and Hashimoto's thyroiditis) in the Japanese population. Diabetes 1998;47(1):128-9.
190. Van der Auwera BJ, Vandewalle CL, Schuit FC, Winnock F, De Leeuw IH, Van Imschoot S, et al. CTLA-4 gene polymorphism confers susceptibility to insulin-dependent diabetes mellitus (IDDM) independently from age and from other genetic or immune disease markers. The Belgian Diabetes Registry. Clin Exp Immunol 1997;110(1):98-103.
191. Marron MP, Raffel LJ, Garchon HJ, Jacob CO, Serrano-Rios M, Martinez Larrad MT, et al. Insulin-dependent diabetes mellitus (IDDM) is associated with CTLA4 polymorphisms in multiple ethnic groups. Hum Mol Genet 1997;6(8):1275-82.
192. Linsley PS, Golstein P. Lymphocyte activation: T-cell regulation by CTLA-4. Curr Biol 1996;6(4):398-400.
193. Walunas TL, Lenschow DJ, Bakker CY, Linsley PS, Freeman GJ, Green JM, et al. CTLA-4 can function as a negative regulator of T cell activation. Immunity 1994;1(5):405-13.
194. Karandikar NJ, Vanderlugt CL, Walunas TL, Miller SD, Bluestone JA. CTLA-4: a negative regulator of autoimmune disease. J Exp Med 1996;184(2):783-8.
195. Waterhouse P, Penninger JM, Timms E, Wakeham A, Shahinian A, Lee KP, et al. Lymphoproliferative disorders with early lethality in mice deficient in CTLA-4. Science 1995;270(5238):985-8.
196. Tivol EA, Borriello F, Schweitzer AN, Lynch WP, Bluestone JA, Sharpe AH. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. Immunity 1995;3(5):541-7.
197. Walunas TL, Bakker CY, Bluestone JA. CTLA-4 ligation blocks CD28-dependent T cell activation. J Exp Med 1996;183(6):2541-50.
198. Sheffield VC, Stone EM, Carmi R. Use of isolated inbred human populations for identification of disease genes. Trends Genet 1998;14(10):391-6.
199. Karam JH, Lewitt PA, Young CW, Nowlain RE, Frankel BJ, Fujiya H, et al. Insulinopenic diabetes after rodenticide (Vacor) ingestion: a unique model of acquired diabetes in man. Diabetes 1980;29(12):971-8.
200. Taniguchi H, Yamashiro Y, Chung MY, Hara Y, Ishihara K, Ejiri K, et al. Vacor inhibits insulin release from islets in vitro. J Endocrinol Invest 1989;12(4):273-5.
201. Wilson GL, Gaines KL. Effects of the rodenticide Vacor on cultured rat pancreatic beta cells. Toxicol Appl Pharmacol 1983;68(3):375-9.
202. Kuttler B, Schneider E. Diabetes mellitus in mice induced by multiple subdiabetogenic doses of streptozotocin: age and sex dependence. Acta Biol Med Ger 1982;41(12):1199-1202.
203. Hoftiezer V, Carpenter AM. Comparison of streptozotocin and alloxan-induced diabetes in the rat, including volumetric quantitation of the pancreatic islets. Diabetologia 1973;9(3):178-84.
204. Weber C, Pernis B, Ting W, Rosenkrantz K, Reemtsma K. Murine streptozotocin diabetes: influences of the major histocompatibility complex, genetic background and blood transfusion. Diabetologia 1984;27 Suppl:160-2.

205. McEvoy RC, Andersson J, Sandler S, Hellerstrom C. Multiple low-dose streptozotocin-induced diabetes in the mouse. Evidence for stimulation of a cytotoxic cellular immune response against an insulin-producing beta cell line. J Clin Invest 1984;74(3):715-22.
206. Harris HF. A case of diabetes mellitus quickly following mumps. Boston Med Surg J 1899;140:465-469.
207. Yoon JW, Austin M, Onodera T, Notkins AL. Isolation of a virus from the pancreas of a child with diabetic ketoacidosis. N Engl J Med 1979;300(21):1173-9.
208. Champsaur H, Dussaix E, Samolyk D, Fabre M, Bach C, Assan R. Diabetes and Coxsackie virus B5 infection. Lancet 1980;1(8162):251.
209. Ahmad N, Abraham AA. Pancreatic isleitis with coxsackie virus B5 infection. Hum Pathol 1982;13(7):661-2.
210. Gladisch R, Hofmann W, Waldherr R. Myocarditis and insulitis following coxsackie virus infection. Z Kardiol 1976;65(10):837-49.
211. Foulis AK, McGill M, Farquharson MA, Hilton DA. A search for evidence of viral infection in pancreases of newly diagnosed patients with IDDM. Diabetologia 1997;40(1):53-61.
212. Pak CY, Cha CY, Rajotte RV, McArthur RG, Yoon JW. Human pancreatic islet cell specific 38 kilodalton autoantigen identified by cytomegalovirus-induced monoclonal islet cell autoantibody. Diabetologia 1990;33(9):569-72.
213. Onodera T, Suzuki H, Toniolo A, Notkins AL. Virus-induced diabetes: cytomegalovirus and multiple environmental insults. Diabetologia 1983;24(3):218-9.
214. Numazaki K, Goldman H, Seemayer TA, Wong I, Wainberg MA. Infection by human cytomegalovirus and rubella virus of cultured human fetal islets of Langerhans. In Vivo 1990;4(1):49-54.
215. Jenson AB, Rosenberg HS. Multiple viruses in diabetes mellitus. Prog Med Virol 1984;29:197-217.
216. Yoon JW. The role of viruses and environmental factors in the induction of diabetes. Curr Top Microbiol Immunol 1991;164:95-123.
217. Gamble DR, Taylor KW, Cumming H. Coxsackie viruses and diabetes mellitus. Br Med J 1973;4(887):260-2.
218. Szopa TM, Titchener PA, Portwood ND, Taylor KW. Diabetes mellitus due to viruses--some recent developments. Diabetologia 1993;36(8):687-95.
219. Cudworth AG, White GB, Woodrow JC, Gamble DR, Lendrum R, Bloom A. Aetiology of juvenile-onset diabetes. A prospective study. Lancet 1977;1(8008):385-8.
220. Hyoty H, Hiltunen M, Knip M, Laakkonen M, Vahasalo P, Karjalainen J, et al. A prospective study of the role of coxsackie B and other enterovirus infections in the pathogenesis of IDDM. Childhood Diabetes in Finland (DiMe) Study Group. Diabetes 1995;44(6):652-7.
221. Field LL, McArthur RG, Shin SY, Yoon JW. The relationship between Coxsackie-B-virus-specific IgG responses and genetic factors (HLA-DR, GM, KM) in insulin-dependent diabetes mellitus. Diabetes Res 1987;6(4):169-73.
222. Friman G, Fohlman J, Frisk G, Diderholm H, Ewald U, Kobbah M, et al. An incidence peak of juvenile diabetes. Relation to Coxsackie B virus immune response. Acta Paediatr Scand Suppl 1985;320:14-9.
223. Frisk G, Fohlman J, Kobbah M, Ewald U, Tuvemo T, Diderholm H, et al. High frequency of Coxsackie-B-virus-specific IgM in children developing type I diabetes during a period of high diabetes morbidity. J Med Virol 1985;17(3):219-27.
224. Frisk G, Diderholm H. Antibody responses to different strains of coxsackie B4 virus in patients with newly diagnosed type I diabetes mellitus or aseptic meningitis. J Infect 1997;34(3):205-10.

225. Ray CG, Palmer JP, Crossley JR, Williams RH. Coxsackie B virus antibody responses in juvenile-onset diabetes mellitus. Clin Endocrinol (Oxf) 1980;12(4):375-8.
226. Clements GB, Galbraith DN, Taylor KW. Coxsackie B virus infection and onset of childhood diabetes. Lancet 1995;346(8969):221-3.
227. Andreoletti L, Hober D, Hober-Vandenberghe C, Belaich S, Vantyghem MC, Lefebvre J, et al. Detection of coxsackie B virus RNA sequences in whole blood samples from adult patients at the onset of type I diabetes mellitus. J Med Virol 1997;52(2):121-7.
228. Dahlquist G, Frisk G, Ivarsson SA, Svanberg L, Forsgren M, Diderholm H. Indications that maternal coxsackie B virus infection during pregnancy is a risk factor for childhood-onset IDDM. Diabetologia 1995;38(11):1371-3.
229. Kang Y, Chatterjee NK, Nodwell MJ, Yoon JW. Complete nucleotide sequence of a strain of coxsackie B4 virus of human origin that induces diabetes in mice and its comparison with nondiabetogenic coxsackie B4 JBV strain. J Med Virol 1994;44(4):353-61.
230. Szopa TM, Gamble DR, Taylor KW. Biochemical changes induced by Coxsackie B4 virus in short-term culture of mouse pancreatic islets. Biosci Rep 1985;5(1):63-9.
231. Horwitz MS, Bradley LM, Harbertson J, Krahl T, Lee J, Sarvetnick N. Diabetes induced by Coxsackie virus: initiation by bystander damage and not molecular mimicry. Nat Med 1998;4(7):781-5.
232. Solimena M, De Camilli P. Coxsackieviruses and diabetes. Nat Med 1995;1(1):25-26.
233. Bach JM, Otto H, Jung G, Cohen H, Boitard C, Bach JF, et al. Identification of mimicry peptides based on sequential motifs of epitopes derived from 65-kDa glutamic acid decarboxylase. Eur J Immunol 1998;28(6):1902-10.
234. Conrad B, Weissmahr RN, Boni J, Arcari R, Schupbach J, Mach B. A human endogenous retroviral superantigen as candidate autoimmune gene in type I diabetes. Cell 1997;90(2):303-13.
235. Lanzavecchia A. How can cryptic epitopes trigger autoimmunity? J Exp Med 1995;181(6):1945-8.
236. Virtanen SM, Rasanen L, Aro A, Lindstrom J, Sippola H, Lounamaa R, et al. Infant feeding in Finnish children less than 7 yr of age with newly diagnosed IDDM. Childhood Diabetes in Finland Study Group. Diabetes Care 1991;14(5):415-7.
237. Virtanen SM, Rasanen L, Ylonen K, Aro A, Clayton D, Langholz B, et al. Early introduction of dairy products associated with increased risk of IDDM in Finnish children. The Childhood in Diabetes in Finland Study Group. Diabetes 1993;42(12):1786-90.
238. Kostraba JN, Cruickshanks KJ, Lawler-Heavner J, Jobim LF, Rewers MJ, Gay EC, et al. Early exposure to cow's milk and solid foods in infancy, genetic predisposition, and risk of IDDM. Diabetes 1993;42(2):288-95.
239. Gerstein HC. Cow's milk exposure and type I diabetes mellitus. A critical overview of the clinical literature. Diabetes Care 1994;17(1):13-9.
240. Saukkonen T, Savilahti E, Vaarala O, Virtala ET, Tuomilehto J, Akerblom HK. Children with newly diagnosed IDDM have increased levels of antibodies to bovine serum albumin but not to ovalbumin. Childhood Diabetes in Finland Study Group. Diabetes Care 1994;17(9):970-6.
241. Saukkonen T, Virtanen SM, Karppinen M, Reijonen H, Ilonen J, Rasanen L, et al. Significance of cow's milk protein antibodies as risk factor for childhood IDDM: interactions with dietary cow's milk intake and HLA- DQB1 genotype. Childhood Diabetes in Finland Study Group. Diabetologia 1998;41(1):72-8.
242. Borch-Johnsen K, Joner G, Mandrup-Poulsen T, Christy M, Zachau-Christiansen B, Kastrup K, et al. Relation between breast-feeding and incidence rates of

insulin- dependent diabetes mellitus. A hypothesis. Lancet 1984;2(8411):1083-6.
243. Kyvik KO, Green A, Svendsen A, Mortensen K. Breast feeding and the development of type 1 diabetes mellitus. Diabet Med 1992;9(3):233-5.
244. Mayer EJ, Hamman RF, Gay EC, Lezotte DC, Savitz DA, Klingensmith GJ. Reduced risk of IDDM among breast-fed children. The Colorado IDDM Registry. Diabetes 1988;37(12):1625-32.
245. Meloni T, Marinaro AM, Mannazzu MC, Ogana A, La Vecchia C, Negri E, et al. IDDM and early infant feeding. Sardinian case-control study. Diabetes Care 1997;20(3):340-2.
246. Norris JM, Beaty B, Klingensmith G, Yu L, Hoffman M, Chase HP, et al. Lack of association between early exposure to cow's milk protein and beta-cell autoimmunity. Diabetes Autoimmunity Study in the Young (DAISY). Jama 1996;276(8):609-14.
247. Bodington MJ, McNally PG, Burden AC. Cow's milk and type 1 childhood diabetes: ni increase in risk. Diabet Med 1994;11(7):663-665.
248. Norris JM, Scott FW. A meta-analysis of infant diet and isulin-dependent diabetes mellitus: do biases play a role? Epidemiology 1996;7:87-92.
249. Karjalainen J, Martin JM, Knip M, Ilonen J, Robinson BH, Savilahti E, et al. A bovine albumin peptide as a possible trigger of insulin-dependent diabetes mellitus. N Engl J Med 1992;327(5):302-7.
250. Hammond-McKibben D, Dosch HM. Cow's milk, bovine serum albumin, and IDDM: Can we settle the controversies. Diabetes care 1997;20(5):897-901.
251. Virtanen SM, Hypponen E, Laara E, Vahasalo P, Kulmala P, Savola K, et al. Cow's milk consumption, disease-associated autoantibodies and type 1 diabetes mellitus: a follow-up study in siblings of diabetic children. Childhood Diabetes in Finland Study Group. Diabet Med 1998;15(9):730-8.
253. Lampasona V, Ferrari M, Bosi E, Pastore MR, Bingley PJ, Bonifacio E. Sera from patients with IDDM and healthy individuals have antibodies to ICA69 on western blots but do not immunoprecipitate liquid phase antigen. J Autoimmun 1994;7(5):665-74.
254. Welsh CJ, Hanglow AC, Conn P, Coombs RR. Comparison of the arthritogenic properties of dietary cow's milk, egg albumin and soya milk in experimental animals. Int Arch Allergy Appl Immunol 1986;80(2):192-9.
255. Vaarala O, Knip M, Paronen J, Hamalainen AM, Muona P, Vaatainen M, et al. Cow's milk formula feeding induces primary immunization to insulin in infants at genetic risk for type 1 diabetes. Diabetes 1999;48(7):1389-94.
256. Cavallo MG, Fava D, Monetini L, Barone F, Pozzilli P. Cell-mediated immune response to beta casein in recent- onset insulin-dependent diabetes: implications for disease pathogenesis. Lancet 1996;348(9032):926-928.
257. Kolb H, Pozzilli P. Cow's milk and type I diabetes: the gut immune system deserves attention. Immunol Today 1999;20(3):108-110.
258. Elliott RB, Harris DP, Hill JP, Bibby NJ, Wasmuth HE. Type I (insulin dependent) diabetes mellitus and cow milk: casein variant consumption. Diabetologia 1999;42(3):292-296.
259. Pozzilli P, Bottazzo GF. Coffee or sugar. Which is to blame in IDDM?. Diabetes Care 1991;14(2):144-5.
260. Virtanen SM, Rasanen L, Aro A, Ylonen K, Lounamaa R, Akerblom HK, et al. Is children's or parents' coffee or tea consumption associated with the risk for type 1 diabetes mellitus in children? Childhood Diabetes in Finland Study Group. Eur J Clin Nutr 1994;48(4):279-85.
261. Parslow RC, McKinney PA, Law GR, Staines A, Williams R, Bodansky HJ. Incidence of childhood diabetes mellitus in Yorkshire, northern England, is associated with nitrate in drinking water: an ecological analysis. Diabetes Care 1997;20(10):1547-52.

262. Virtanen SM, Jaakkola L, Rasanen L, Ylonen K, Aro A, Lounamaa R, et al. Nitrate and nitrite intake and the risk for type 1 diabetes in Finnish children. Childhood Diabetes in Finland Study Group. Diabet Med 1994;11(7):656-62.
263. Kostraba JN, Gay EC, Rewers M, Hamman RF. Nitrate levels in community drinking waters and risk of IDDM. An ecological analysis. Diabetes Care 1992;15(11):1505-8.
264. Association AD. Prevention of Type 1 Diabetes Mellitus. Diabetes Care 1999;22(S1):S102.
265. Avery L, Borsey DQ, Greene SA, Gummerson I, Hayes R, Kerr D, et al. British Diabetic Association guidelines on genetic and immune screening for type 1 diabetes mellitus. Diabet Med 1998;15(8):643.
266. Dahlquist GG. Primary and Secondary Prevention Strategies of Pre-type 1 Diabetes. Diabetes Care 1999;22(S2).
267. Buzzetti R, Nistico L, Osborn JF, Giovannini C, Chersi A, Sorrentino R. HLA-DQA1 and DQB1 gene polymorphisms in type I diabetic patients from central Italy and their use for risk prediction. Diabetes 1993;42:1173-1178.
268. Cucca F, Muntoni F, Lampis R, Frau F, Argiolas L, Silvetti M, et al. Combinations of specific DRB1, DQA1, DQB1 haplotypes are associated with insulin-dependent diabetes mellitus in Sardinia. Hum Immunol 1993;37(2):85-94.
269. Sanjeevi CB, Lybrand TP, Landin-Olsson M, Kockum I, Dahlquist G, Hagopian WA, et al. Analysis of antibody markers, DRB1, DRB5, DQA1 and DQB1 genes and modeling of DR2 molecules in DR2-positive patients with insulin-dependent diabetes mellitus. Tissue Antigens 1994;44(2):110-9.
270. She X. Susceptibility to type 1 diabetes: HLA-DQ and DR revisited. Immunol Today 1996;17(7):323-329.
271. Pugliese A, Gianani R, Moromisato R, Awdeh ZL, Alper CA, Erlich HA, et al. HLA-DQB1*0602 is associated with dominant protection from diabetes even among islet cell antibody-positive first-degree relatives of patients with IDDM. Diabetes 1995;44(6):608-13.
272. Gianani R, Verge CF, Moromisatogianani RI, Yu L, Zhan YJ, Pugliese A, et al. Limited loss of tolerance to islet autoantigens in ICA+ first degree relatives of patients with type i diabetes expressing the HLA DQB1*0602 allele. J Autoimmun 1996;9(3):423-425.
273. Bonifacio E, Bingley PJ. Islet autoantibodies and their use in predicting insulin-dependent diabetes. Acta Diabetol 1997;34(3):185-93.
274. Bingley PJ, Bonifacio E, Williams AJ, Genovese S, Bottazzo GF, Gale EA. Prediction of IDDM in the general population: strategies based on combinations of autoantibody markers. Diabetes 1997;46(11):1701-10.
275. Kulmala P, Savola K, Petersen JS, Vahasalo P, Karjalainen J, Lopponen T, et al. Prediction of insulin-dependent diabetes mellitus in siblings of children with diabetes. A population-based study. The Childhood Diabetes in Finland Study Group. J Clin Invest 1998;101(2):327-36.
276. Kockum I, Lernmark A, Dahlquist G, Falorni A, Hagopian WA, Landinolsson M, et al. Genetic and immunological findings in patients with newly diagnosed insulin-dependent diabetes mellitus. Horm Metab Res 1996;28(7):344-347.
277. Gorus FK, Goubert P, Semakula C, Vandewalle CL, De Schepper J, Scheen A, et al. IA-2-autoantibodies complement GAD65-autoantibodies in new-onset IDDM patients and help predict impending diabetes in their siblings. The Belgian Diabetes Registry. Diabetologia 1997;40(1):95-9.
278. Botazzo GF, Florin-Christensen A, Doniach D. Islet cell antibodies in diabetes mellitus with autoimmune poyendocrine deficiency. Lancet 1974;ii:1279.
279. Marner B, Lernmark A, Nerup J, Molenaar JL, Tuk CW, Bruining GJ. Analysis of islet cell antibodies on frozen sections of human pancreas. Diabetologia 1983;25(2):93-6.

280. Boitard C, Bonifacio E, Bottazzo GF, Gleichmann H, Molenaar J. Immunology and Diabetes Workshop: report on the Third International (Stage 3) Workshop on the Standardisation of Cytoplasmic Islet Cell Antibodies. Held in New York, New York, October 1987. Diabetologia 1988;31(7):451-2.
281. Lernmark A, Molenaar JL, van Beers WA, Yamaguchi Y, Nagataki S, Ludvigsson J, et al. The Fourth International Serum Exchange Workshop to standardize cytoplasmic islet cell antibodies. The Immunology and Diabetes Workshops and Participating Laboratories. Diabetologia 1991;34(7):534-5.
282. Greenbaum CJ, Palmer JP, Nagataki S, Yamaguchi Y, Molenaar JL, Van Beers WA, et al. Improved specificity of ICA assays in the Fourth International Immunology of Diabetes Serum Exchange Workshop. Diabetes 1992;41(12):1570-4.
283. Brogren CH, Baekkeskov S, Dyrberg T, Lernmark A, Marner B, Nerup J, et al. Role of islet cell antibodies in the pathogenesis of type I diabetes. Curr Probl Clin Biochem 1983;12:65-85.
284. Papadopoulos G, Lernmark A. Islet cell antibodies and cellular immunity in human diabetes. Behring Inst Mitt 1984;75:50-7.
285. Bruining G, Molenaar J, Tuk C, Lindeman J, Bruining H, Marner B. Clinical time-course and characteristics of islet cell cytoplasmic antibodies in childhood diabetes. Diabetologia 1984;1984(26):24-29.
286. Hagglof B, Rabinovitch A, Mackay P, Huen A, Rubenstein AH, Marner B, et al. Islet cell and other organ-specific autoantibodies in healthy first-degree relatives to insulin-dependent. Acta Paediatr Scand 1986;75(4):611-8.
287. Roll U, Ziegler AG. Combined antibody screening for improved prediction of IDDM--modern strategies. Exp Clin Endocrinol Diabetes 1997;105(1):1-14.
288. Palmer JP, Asplin CM, Clemons P, Lyen K, Tatpati O, Raghu PK, et al. Insulin antibodies in insulin-dependent diabetics before insulin treatment. Science 1983;222(4630):1337-9.
289. Greenbaum CJ, Wilkin TJ, Palmer JP. Fifth International Serum Exchange Workshop for Insulin Autoantibody (IAA) Standardization. The Immunology and Diabetes Workshops and participating laboratories. Diabetologia 1992;35(8):798-800.
290. Levy-Marchal C, Bridel MP, Sodoyez-Goffaux F, Koch M, Tichet J, Czernichow P, et al. Superiority of radiobinding assay over ELISA for detection of IAAs in newly diagnosed type I diabetic children. Diabetes Care 1991;14(1):61-3.
291. Williams AJ, Bingley PJ, Bonifacio E, Palmer JP, Gale EA. A novel micro-assay for insulin autoantibodies. J Autoimmun 1997;10(5):473-8.
292. Naserke HE, Dozio N, Ziegler AG, Bonifacio E. Comparison of a novel micro-assay for insulin autoantibodies with the conventional radiobinding assay. Diabetologia 1998;41(6):681-3.
293. Arslanian SA, Becker DJ, Rabin B, Atchison R, Eberhardt M, Cavender D, et al. Correlates of insulin antibodies in newly diagnosed children with insulin-dependent diabetes before insulin therapy. Diabetes 1985;34(9):926-30.
294 Vardi P, Ziegler AG, Mathews JH, Dib S, Keller RJ, Ricker AT, et al. Concentration of insulin autoantibodies at onset of type I diabetes: inverse log-linear correlation with age. Diabetes Care 1988;11:736-739.
295. Hagopian WA, Sanjeevi CB, Kockum I, Landin-Olsson M, Karlsen AE, Sundkvist G, et al. Glutamate decarboxylase-, insulin-, and islet cell-antibodies and HLA typing to detect diabetes in a general population-based study of Swedish children. J Clin Invest 1995;95(4):1505-11.
296. Roll U, Christie MR, Fuchtenbusch M, Payton MA, Hawkes CJ, Ziegler AG. Perinatal autoimmunity in offspring of diabetic parents: the german multicenter BABY-DIAB study: detection of humoral immune responses to islet antigens in early childhood. Diabetes 1996;45(7):967-973.

297. Ziegler AG, Hillebrand B, Rabl W, Mayrhofer M, Hummel M, Mollenhauer U, et al. On the appearance of islet associated autoimmunity in offspring of diabetic mothers: a prospective study from birth. Diabetologia 1993;36(5):402-8.
298. Vahasalo P, Knip M, Karjalainen J, Tuomilehto-Wolf E, Lounamaa R, Akerblom HK. Islet cell-specific autoantibodies in children with insulin-dependent diabetes mellitus and their siblings at clinical manifestation of the disease. Childhood Diabetes in Finland Study Group. Eur J Endocrinol 1996;135(6):689-95.
299. Castano L, Ziegler AG, Ziegler R, Shoelson S, Eisenbarth GS. Characterization of insulin autoantibodies in relatives of patients with type I diabetes. Diabetes 1993;42(8):1202-9.
300. Verge CF, Gianani R, Kawasaki E, Yu L, Pietropaolo M, Jackson RA, et al. Prediction of type I diabetes in first-degree relatives using a combination of insulin, GAD, and ICA512bdc/IA-2 autoantibodies. Diabetes 1996;45(7):926-33.
301. Baekkeskov S, Nielsen JH, Marner B, Bilde T, Ludvigsson J, Lernmark A. Autoantibodies in newly diagnosed diabetic children immunoprecipitate human pancreatic islet cell proteins. Nature 1982;298(5870):167-9.
302. Bieg S, Seissler J, Herberg L, Northemann W, Scherbaum WA. GAD65 is recognized by T-cells, but not by antibodies from NOD-mice. Autoimmunity 1994;17(3):189-94.
303. Eisenbarth GS. Mouse or man. Is GAD the cause of type I diabetes?.. Diabetes Care 1994;17(6):605-7.
304. Petersen JS, Dyrberg T, Karlsen AE, Molvig J, Michelsen B, Nerup J, et al. Glutamic acid decarboxylase (GAD65) autoantibodies in prediction of beta-cell function and remission in recent-onset IDDM after cyclosporin treatment. The Canadian-European Randomized Control Trial Group. Diabetes 1994;43(11):1291-6.
305. Schloot NC, Daniel D, Norburyglaser M, Wegmann DR. Peripheral t cell clones from NOD mice specific for GAD65 peptides: lack of islet responsiveness or diabetogenicity. J Autoimmun 1996;9(3):357-363.
306. Verge CF, Stenger D, Bonifacio E, Colman PG, Pilcher C, Bingley PJ, et al. Combined use of autoantibodies (IA-2 autoantibody, GAD autoantibody, insulin autoantibody, cytoplasmic islet cell antibodies) in type 1 diabetes: Combinatorial Islet Autoantibody Workshop. Diabetes 1998;47(12):1857-66.
307. Kaufman DL. Spontaneous loss of T-cell tolerance to glutamic acid decarboxylase in murine insulin-dependent diabetes. Nature 1993;366:69-72.
308. Tish R, Yang X, Singer S, Liblau R, Fugger L, McDevitt H. Immune response to glutamic acid decarboxylase correlates with insulitis in NOD mice. Nature 1993;366:72-75.
309. Erlander MG. Two genes encode distinct glutamate decarboxylases. Neuron 1991;7:91-100.
310. Karlsen AE, Hagopian WA, Grubin CE, Dube S, Disteche CM, Adler DA, et al. Cloning and primary structure of a human islet isoform of glutamic acid decarboxylase from chromosome 10. Proc Natl Acad Sci U S A 1991;88(19):8337-41.
311. Bu DF, Erlander MG, Hitz BC, Tillakaratne NJ, Kaufman DL, Wagner-McPherson CB, et al. Two human glutamate decarboxylases, 65-kDa GAD and 67-kDa GAD, are each encoded by a single gene. Proc Natl Acad Sci U S A 1992;89(6):2115-9.
312. Celotti F, Moore GD, Rovescalli AC, Solimena M, Negri-Cesi P, Racagni G. The GABAergic system in the rat oviduct: presence, endocrine regulation and possible localization. Pharmacol Res 1989;21(1):103-4.
313. Erdo SL, Joo F, Wolff JR. Immunohistochemical localization of glutamate decarboxylase in the rat oviduct and ovary: further evidence for non-neural GABA systems. Cell Tissue Res 1989;255(2):431-4.

314. Cram DS, Barnett LD, Joseph JL, Harrison LC. Cloning and partial nucleotide sequence of human glutamic acid decarboxylase cDNA from brain and pancreatic islets. Biochem Biophys Res Commun 1991;176(3):1239-44.
315. Christgau S, Schierbeck H, Aanstoot HJ, Aagaard L, Begley K, Kofod H, et al. Pancreatic beta cells express two autoantigenic forms of glutamic acid decarboxylase, a 65-kDa hydrophilic form and a 64-kDa amphiphilic form which can be both membrane-bound and. J Biol Chem 1991;266(31):21257-64.
316. Cram D, Faulkner-Jones B, Kun J, Harrison L. Glutamic acid decarboxylase-67 (GAD67): expression relative to GAD65 in human islets and mapping of autoantibody epitopes. Endocrinology 1995;136(3):1111-1119.
317. Kim J, Richter W, Aanstoot H, Shi Y, Fu Q, Rajotte R, et al. Differential expression of GAD65 and GAD67 in human, rat, and mouse pancreatic islets. Diabetes 1993;42:1799-808.
318. Petersen JS, Russel S, Marshall MO, Kofod H, Buschard K, Cambon N, et al. Differential expression of glutamic acid decarboxylase in rat and human islets. Diabetes 1993;42(3):484-95.
319. Seissler J, Amann J, Mauch L, Haubruck H, Wolfahrt S, Bieg S, et al. Prevalence of autoantibodies to the 65- and 67-kD isoforms of glutamate decarboxylase in insulin-dependent diabetes mellitus. J Clin Invest 1993;92(3):1394-9.
320. Richter W, Shi Y, Baekkeskov S. Autoreactive epitopes defined by diabetes-associated human monoclonal antibodies are localized in the middle and C-terminal domains of the smaller form of glutamate decarboxylase. Proc Natl Acad Sci U S A 1993;90(7):2832-6.
321. Richter W, Seissler J, Northemann W, Wolfahrt S, Meinck HM, Scherbaum WA. Cytoplasmic islet cell antibodies recognize distinct islet antigens in IDDM but not in stiff man syndrome. Diabetes 1993;42(11):1642-8.
322. Hagopian WA, Michelsen B, Karlsen AE, Larsen F, Moody A, Grubin CE, et al. Autoantibodies in IDDM primarily recognize the 65,000-M(r) rather than the 67,000-M(r) isoform of glutamic acid decarboxylase. Diabetes 1993;42(4):631-6.
323. Ujihara N, Daw K, Gianani R, Boel E, Yu L, Powers AC. Identification of glutamic acid decarboxylase autoantibody heterogeneity and epitope regions in type I diabetes. Diabetes 1994;43(8):968-75.
324. Falorni A, Ackefors M, Carlberg C, Daniels T, Persson B, Robertson J, et al. Diagnostic sensitivity of immunodominant epitopes of glutamic acid decarboxylase (GAD65) autoantibodies in childhood IDDM. Diabetologia 1996;39(9):1091-8.
325. Daw K, Ujihara N, Atkinson M, Powers AC. Glutamic acid decarboxylase autoantibodies in stiff-man syndrome and insulin-dependent diabetes mellitus exhibit similarities and differences in epitope recognition. J Immunol 1996;156(2):818-25.
326. Daw K, Powers A. Two distinct glutamic acid decarboxylase auto-antibody specificities in IDDM target different epitopes. Diabetes 1995;44(2):216-220.
327. Tuomi T, Rowley MJ, Knowles WJ, Chen QY, McAnally T, Zimmet PZ, et al. Autoantigenic properties of native and denatured glutamic acid decarboxylase: evidence for a conformational epitope. Clin Immunol Immunopathol 1994;71(1):53-9.
328. Bjork E, Velloso LA, Kampe O, Karlsson FA. GAD autoantibodies in IDDM, stiff-man syndrome, and autoimmune polyendocrine syndrome type I recognize different epitopes. Diabetes 1994;43(1):161-5.
329. Kim J, Namchuk M, Buchawan T, Fu Q, Jaffe M, Shi Y, et al. Higher autoantibody levels and regognition of a linear NH2-terminal epitope in the autoantigen GAD65, distinguish stiff-man syndrome syndrome from insulin dependent diabetes mellitus. J Exp Med 1994;180(2):595-606.

330. Li L, Hagopian WA, Brashear HR, Daniels T, Lernmark A. Identification of autoantibody epitopes of glutamic acid decarboxylase in stiff-man syndrome patients. J Immunol 1994;152(2):930-4.
331. Dinkel K, Meinck HM, Jury KM, Karges W, Richter W. Inhibition of gamma-aminobutyric acid synthesis by glutamic acid decarboxylase autoantibodies in stiff-man syndrome. Ann Neurol 1998;44(2):194-201.
332. Schmidli RS, Colman PG, Bonifacio E, Bottazzo GF, Harrison LC. High level of concordance between assays for glutamic acid decarboxylase antibodies. The First International Glutamic Acid Decarboxylase Antibody Workshop. Diabetes 1994;43(8):1005-9.
333. Grubin CE, Daniels T, Toivola B, Landin-Olsson M, Hagopian WA, Li L, et al. A novel radioligand binding assay to determine diagnostic accuracy of isoform-specific glutamic acid decarboxylase antibodies in childhood IDDM. Diabetologia 1994;37(4):344-50.
334. Falorni A, Ortqvist E, Persson B, Lernmark A. Radioimmunoassays for glutamic acid decarboxylase (GAD65) and GAD65 autoantibodies using 35S or 3H recombinant human ligands. J Immunol Methods 1995;186(1):89-99.
335. Petersen JS, Hejnaes KR, Moody A, Karlsen AE, Marshall MO, Hoier-Madsen M, et al. Detection of GAD65 antibodies in diabetes and other autoimmune diseases using a simple radioligand assay. Diabetes 1994;43(3):459-67.
336. Baron EJ, Weber DE, Weide LG. Lack of agreement among two commercial enzyme-linked immunosorbent antibody assays and a conventional immunofluorescence-based method for detecting islet cell autoantibodies. Clin Diagn Lab Immunol 1996;3(4):429-31.
337. Kohnert KD, Besch W, Rjasanowski I. Discordant results in the assay of cytoplasmic islet cell autoantibodies with ELISA and immunohistochemical techniques. Eur J Clin Chem Clin Biochem 1995;33(7):447-9.
338. Mehta HB, Vold BS, Minkin S, Ullman EF. DELISA: sensitive nonisotopic assay for GAD65 autoantibodies, a key risk-assessment marker for insulin-dependent diabetes mellitus. Clin Chem 1996;42(2):263-9.
339. Tiberti C, Falorni A, Torresi P, Vecci E, Anastasi E, Dotta F, et al. A new solid-phase radioimmunoassay to detect anti-GAD65 autoantibodies. J Immunol Methods 1997;207(2):107-13.
340. Vandewalle CL, Coeckelberghs MI, De Leeuw IH, Du Caju MV, Schuit FC, Pipeleers DG, et al. Epidemiology, clinical aspects, and biology of IDDM patients under age 40 years. Comparison of data from Antwerp with complete ascertainment with data from Belgium with 40% ascertainment. The Belgian Diabetes Registry. Brit Med J 1997;315(7107):524-8.
341. Savola K, Sabbah E, Kulmala P, Vahasalo P, Ilonen J, Knip M. Autoantibodies associated with Type I diabetes mellitus persist after diagnosis in children. Diabetologia 1998;41(11):1293-7.
342. Ludvigsson J, Hellstrom S. Autoantibodies in relation to residual insulin secretion in children with IDDM. Diabetes Res Clin Pract 1997;35(2-3):81-9.
343. Komulainen J, Knip M, Lounamaa R, Vahasalo P, Karjalainen J, Sabbah E, et al. Poor beta-cell function after the clinical manifestation of type 1 diabetes in children initially positive for islet cell specific autoantibodies. The Childhood Diabetes in Finland Study Group. Diabet Med 1997;14(7):532-7.
344. Sabbah E, Kulmala P, Veijola R, Vahasalo P, Karjalainen J, Tuomilehto-Wolf E, et al. Glutamic acid decarboxylase antibodies in relation to other autoantibodies and genetic risk markers in children with newly diagnosed insulin-dependent diabetes. Childhood Diabetes in Finland Study Group. J Clin Endocrinol Metab 1996;81(7):2455-9.
345. Hagopian WA, Karlsen AE, Gottsater A, Landin-Olsson M, Grubin CE, Sundkvist G, et al. Quantitative assay using recombinant human islet glutamic acid decarboxylase (GAD65) shows that 64K autoantibody positivity at onset predicts diabetes type. J Clin Invest 1993;91(1):368-74.

346. Bonifacio E, Genovese S, Braghi S, Bazzigaluppi E, Lampasona V, Bingley PJ, et al. Islet autoantibody markers in IDDM: risk assessment strategies yielding high sensitivity. Diabetologia 1995;38(7):816-22.
347. Payton M, Hawkes C, Christie M. Relationship of the 37,000- and 40,000-M(r) tryptic fragments of islet antigens in insulin-dependent diabetes to the protein tyrosine phosphatase-like molecule IA-2 (ICA512). J Clin Invest. 1995;96(3):1506-1511.
348. Lu J, Li Q, Xie H, Chen ZJ, Borovitskaya AE, Maclaren NK, et al. Identification of a second transmembrane protein tyrosine phosphatase, IA-2 beta, as an autoantigen in insulin- dependent diabetes mellitus: precursor of the 37-kda tryptic fragment. Proc Natl Acad Sci USA 1996;93(6):2307-2311.
349. Rabin DU, Pleasic SM, Shapiro JA, Yoo-Warren H, Oles J, Hicks JM, et al. Islet cell antigen 512 is a diabetes-specific islet autoantigen related to protein tyrosine phosphatases. J Immunol 1994;152(6):3183-8.
350. Christie MR, Vohra G, Champagne P, Daneman D, Delovitch TL. Distinct antibody specificities to a 64-kD islet cell antigen in type 1 diabetes as revealed by trypsin treatment. J Exp Med 1990;172(3):789-94.
351. Solimena M, Dirkx R, Jr., Hermel JM, Pleasic-Williams S, Shapiro JA, Caron L, et al. ICA 512, an autoantigen of type I diabetes, is an intrinsic membrane protein of neurosecretory granules. Embo J 1996;15(9):2102-14.
352. Naserke HE, Ziegler AG, Lampasona V, Bonifacio E. Early development and spreading of autoantibodies to epitopes of IA-2 and their association with progression to type 1 diabetes. J Immunol 1998;161(12):6963-9.
353. Gianani R, Rabin DU, Verge CF, Yu L, Babu SR, Pietropaolo M, et al. ICA512 autoantibody radioassay. Diabetes 1995;44(11):1340-4.
354. Schatz DA, Rogers DG, Brouhard BH. Prevention of insulin-dependent diabetes mellitus: an overview of three trials. Cleve Clin J Med 1996;63(5):270-4.
355. Castano L, Russo E, Zhou L, Lipes MA, Eisenbarth GS. Identification and cloning of a granule autoantigen (carboxypeptidase-H) associated with type I diabetes. J Clin Endocrinol Metab 1991;73(6):1197-201.
356. Baekkeskov S, Aanstoot HJ, Fu Q, Jaffe M, Kim J, Quan J, et al. The glutamate decarboxylase and 38KD autoantigens in type 1 diabetes: aspects of structure and epitope recognition. Autoimmunity 1993;15(Suppl):24-6.
357. Bazzigaluppi E, Bonfanti R, Bingley PJ, Bosi E, Bonifacio E. Capillary Whole Blood Measurement of Islet Autoantibodies. Diabetes Care 1999;22(2):275-279.
358. Wiest-Ladenburger U, Hartmann R, Hartmann U, Berling K, Bohm BO, Richter W. Combined analysis and single-step detection of GAD65 and IA2 autoantibodies in IDDM can replace the histochemical islet cell antibody test. Diabetes 1997;46(4):565-71.
359. Christie MR, Roll U, Payton MA, Hatfield EC, Ziegler AG. Validity of screening for individuals at risk for type I diabetes by combined analysis of antibodies to recombinant proteins. Diabetes Care 1997;20(6):965-70.
360. Mcrae BL, Vanderlugt CL, Dalcanto MC, Miller SD. Functional evidence for epitope spreading in the relapsing pathology of experimental autoimmune encephalomyelitis. Journal of Experimental Medicine 1995;182(1):75-85.
361. Kawasaki E, Yu L, Rewers MJ, Hutton JC, Eisenbarth GS. Definition of multiple ICA512/phogrin autoantibody epitopes and detection of intramolecular epitope spreading in relatives of patients with type 1 diabetes. Diabetes 1998;47(5):733-42.
362. Kim J, Namchuk M, Bugawan T, Fu Q, Jaffe M, Shi Y, et al. Higher autoantibody levels and recognition of a linear NH2-terminal epitope in the autoantigen GAD65, distinguish stiff-man syndrome from insulin-dependent diabetes mellitus. J Exp Med 1994;180(2):595-606.

363. Jury KM, Loeffler D, Eiermann TH, Ziegler B, Boehm BO, Richter W. Evidence for somatic mutation and affinity maturation of diabetes associated human autoantibodies to glutamate decarboxylase. J Autoimmun 1996;9(3):371-377.
364. Richter W, Jury K, Loeffler D, Manfras B, Eiermann T, Boehm B. Immunoglobulin variable gene analysis of human autoantibodies reveals antigen-driven immune response to glutamate decarboxylase in type 1 diabetes mellitus. Eur J Immunol. 1995;25(6):1703-12.
365. Luhder F, Schlosser M, Mauch L, Haubruck H, Rjasanowski I, Michaelis D, et al. Autoantibodies against GAD65 rather than GAD67 precede the onset of type 1 diabetes. Autoimmunity 1994;19(2):71-80.
366. Verge CF, Gianani R, Kawasaki E, Yu L, Pietropaolo M, Chase HP, et al. Number of autoantibodies (against insulin, GAD or ICA512/IA2) rather than particular autoantibody specificities determines risk of type I diabetes. J Autoimmun 1996;9(3):379-83.
367. Altobelli E, Chiarelli F, Valenti M, Verrotti A, Tumini S, Di Orio F. Familial risk of type I diabetes in European children. The Eurodiab Ace Study Group and The Eurodiab Ace Substudy 2 Study Group.Incidence of insulin-dependent diabetes mellitus (0-14 years) in the Abruzzo Region, Italy, 1990-1995: results from a population-based register. Diabetologia 1998;41(10):1151-6.
368. Pociot F, Norgaard K, Hobolth N, Andersen O, Nerup J. A nationwide population-based study of the familial aggregation of type 1 (insulin-dependent) diabetes mellitus in Denmark. Danish Study Group of Diabetes in Childhood. Diabetologia 1993;36(9):870-5.
369. Schatz D, Krischer J, Horne G, Riley W, Spillar R, Silverstein J, et al. Islet cell antibodies predict insulin-dependent diabetes in United States school age children as powerfully as in unaffected relatives. J Clin Invest 1994;93(6):2403-7.
370. Knip M, Karjalainen J, Akerblom HK. Islet cell antibodies are less predictive of IDDM among unaffected children in the general population than in sibs of children with diabetes. The Childhood Diabetes in Finland Study Group. Diabetes Care 1998;21(10):1670-3.
371. Akerblom HK, Knip M, Simell O. From pathomechanisms to prediction, prevention and improved care of insulin-dependent diabetes mellitus in children. Ann Med 1997;29(5):383-5.
372. Gale EA. Nicotinamide: potential for the prevention of type 1 diabetes? Horm Metab Res 1996;28(7):361-4.
373. Gale EA. Theory and practice of nicotinamide trials in pre-type 1 diabetes. J Pediatr Endocrinol Metab 1996;9(3):375-9.
374. Gale EA. Molecular mechanisms of beta-cell destruction in IDDM: the role of nicotinamide. Horm Res 1996;45(Suppl 1):39-43.
375. Lazarow A. Protection against alloxan diabetes. Anat Rec 1947;97:353.
376. Yamada K, Nonaka K, Hanafusa T, Miyazaki A, Toyoshima H, Tarui S. Preventive and therapeutic effects of large-dose nicotinamide injections on diabetes associated with insulitis. An observation in nonobese diabetic (NOD) mice. Diabetes 1982;31(9):749-53.
377. Elliott RB, Pilcher CC, Stewart A, Fergusson D, McGregor MA. The use of nicotinamide in the prevention of type 1 diabetes. Ann N Y Acad Sci 1993;696:333-41.
378. Elliott RB, Pilcher CC, Fergusson DM, Stewart AW. A population based strategy to prevent insulin-dependent diabetes using nicotinamide. J Pediatr Endocrinol Metab 1996;9(5):501-9.
379. Gerstein HC, Simpson JR, Atkinson S, Taylor DW, VanderMeulen J. The Diabetes Prevention Trial-Type 1 diabetes (DPT-1): implementation of screening and staging of relatives. DPT-1 Study Group. Feasibility and acceptability of a proposed infant feeding intervention trial for the prevention of type I diabetes. Transplant Proc 1995;27(6):3377.

Chapter 2

The natural course of ß-cell autoimmunity

Chapter 2.1

Fluctuations in GAD_{65} Antibodies After Clinical Diagnosis of IDDM in Young Children

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OBJECTIVE — To investigate whether the presence of GAD antibodies at onset of IDDM correlates to a more aggressive rate of β-cell destruction after clinical onset.

RESEARCH DESIGN AND METHODS — We studied GAD antibodies at onset of disease, after 1 year, and after 6 years in 33 consecutively referred children (mean age 8.08, range 1.7–16.3). In a subset of 11 patients, GAD antibodies were studied very frequently. The correlation between GAD antibodies and clinical parameters, including glycosylated hemoglobin, residual insulin secretion, and insulin dosage, was evaluated.

RESULTS — GAD antibody titers were highly variable. Four patients became GAD antibody positive weeks to years after clinical onset. Other patients switched between testing positive and negative for GAD antibodies shortly after clinical onset. No correlation was found between the presence of GAD antibodies and the rate of β-cell destruction, but patients with high GAD antibody indexes at onset had significantly higher glycosylated hemoglobin levels.

CONCLUSIONS — GAD antibodies at clinical onset do not predict the rate of β-cell destruction in young children with newly diagnosed IDDM. The highly variable GAD antibody levels suggest variation of the autoimmune process.

Antibodies against GAD are detected in ~80% of newly diagnosed patients with IDDM and prediabetic individuals (1,2). Petersen and colleagues (3) showed that the presence of GAD antibodies at clinical onset of IDDM predicted the course of β-cell destruction in adolescent and adult patients. However, β-cell destruction in young children occurs faster, and data on prospective follow-up of GAD antibodies in IDDM in newly diagnosed young children are scarce. We examined the course of GAD antibodies and their correlation with the clinical course of the disease in 33 consecutively referred children with newly diagnosed IDDM.

RESEARCH DESIGN AND METHODS — Thirty-three consecutively referred newly diagnosed children (15 girls, mean age at diagnosis of IDDM 8.08 ± 3.97 [range: 1.7–16.3]), who entered a trial of continuous subcutaneous insulin infusion (CSII) in 1982–1984 (4,5), were studied. Serum samples and clinical data were collected over a 2-year period. Serum samples were available up to a maximum of 8 years of diabetes duration (mean 6 years, range 3–8, due to leaving clinic and difficulties in obtaining new samples). The study was approved by local ethics committees in accordance with the Declaration of Helsinki, and signed informed consent was obtained.

GAD antibodies were tested by immunoprecipitations of BHK (baby hamster kidney) cells which stably expressed human GAD_{65}, followed by SDS-PAGE and fluorography (2,3) The results were correlated to standard negative and positive control sera and expressed as GAD index. Dilution curves showed that this gel method is at least one dilution step more sensitive due to low background levels and unambiguous identification of the GAD doublet band (data not shown). This assay detected 84% GAD positives (n = 150) compared with 80% for the widely applied radioimmunoassay (RIA). GAD antibodies were tested in sera collected at onset, after 1 year, and after 6 years (mean, range 3–8 years) of IDDM duration in all 33 children. The course of GAD antibodies was studied in detail in a subset of 11 children, of whom 12–15 serum samples from the first 2 years of disease were available. In the immunoprecipitations, the interassay variation for the positive control was 12%. A GAD index of 0.16 or lower was negative. This was confirmed by 3 months exposure of gels to X-ray film. Technical disturbances by prozone effects or concentration effects were excluded by dilution experiments and by normal sodium, potassium and total protein levels in sera with high GAD antibody titers (data not shown). All sera were tested at least three times in independent experiments. The endogenous insulin production (24-h urinary C-peptide secretion) and total glycosylated hemoglobin were measured as described (5–7).

Complete data sets were available in 31 patients. Data were analyzed combined and separately for the conventional and the CSII group. For comparisons between groups, Wilcoxon's rank-sum test (SPSS-PC+, SPSS Inc., Chicago, IL) was used. The correlation between GAD antibodies and clinical parameters was tested by regression analysis. Age-corrected C-peptide secretion (ccpep) and GAD indexes were log-transformed to obtain Gaussian-shaped distribution (lnccpep = ln[ccpep + 1] and lnGAD − index = ln[GAD − index + 0.1]).

RESULTS — At onset, 23 (70%) of the

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ccpep, C-peptide secretion; CSII, continuous subcutaneous insulin infusion; RIA, radioimmunoassay.

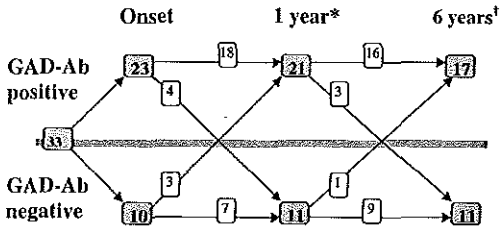

Figure 1—*The course of GAD antibodies positivity/negativity of 33 children with IDDM at onset and after 1 and 6 years of disease duration. Shaded numbers indicate numbers of patients; unshaded numbers indicate numbers of seroconverting patients. *Serum from one patient not available. †Sera from four patients not available for testing.*

children were GAD antibody positive, 10 (30%) patients were negative. The GAD status was independent of age of onset. Figure 1 depicts the variation in the GAD status of the children at onset and after 1 and 6 years. Seventeen of 28 children (59%) tested GAD antibody positive at longer duration of diabetes (mean 6 years). In three of 11 patients tested repeatedly during the first 2 years of disease, we observed a switch from negative to positive (and back) within weeks to months, whereas in other patients high peaks in GAD index were followed by lower titers in consecutive sera tested. One patient converted from GAD antibody negative to weakly positive and back within a month. Another was negative for GAD antibodies at onset of the disease, but positive at 1–2 months and 4–5 months duration, remaining negative during the intervening period. Strikingly, one patient tested negative for GAD antibodies in all serum samples taken during the first 16 months of disease and tested positive in the next serum sample, taken at 56 months of disease duration. In Fig. 2, fluctuations in GAD antibody indexes in the repeatedly tested patients are shown graphically. A significant correlation between the glycosylated hemoglobin at onset and GAD index at onset, but not with GAD status at onset, was found ($R = 0.38$, $P = 0.037$). No other correlations between GAD status or GAD index and clinical parameters (Table 1) at any timepoint were observed. The CSII-treated patients experienced a longer remission period (defined as three successive age-corrected C-peptide measurements of 30% or less) than did the conventional treated patients (8). However, this was not reflected by GAD antibodies.

CONCLUSIONS — Despite intense efforts, blood glucose regulation in young children with IDDM remains unsatisfactory. Endogenous insulin production may facilitate blood glucose regulation. Better understanding of the heterogeneous disease process, also during the honeymoon, may be beneficial for later control. We studied whether the presence of GAD antibodies at clinical onset in young children is correlated to a more aggressive β-cell destruction, as was suggested in adolescents and adults (3). No such correlation was found. In fact, the observed correlation between glycosylated hemoglobin levels and GAD antibodies suggests a less aggressive β-cell autoimmunity in GAD antibody–positive patients, allowing for a longer preclinical period with deranged blood glucose metabolism. Whereas others previously described the yet unexplained phenomenon of persisting GAD antibodies long after clinical onset of IDDM, we identified highly fluctuating titers. The previously described heterogeneity of autoimmune destruction in the human pancreas (9) and functional heterogeneity of β-cells, which may result in different levels of GAD_{65} expression and blood glucose regulation (10), may account for the observed fluctuations. Excluding technical failures, it is conceivable that similar fluctuations exist in the preclinical phase as has been found for ICAs (11,12). However, in these studies once positive samples did not become negative. Our data warrant repetitive testing of GAD antibodies in IDDM prediction.

We prefer the traditional gel method for delicate analyses. RIA, widely used in prediction studies, has the disadvantage of relatively high background levels with concomitant problems of threshold setting. The gel method has a low background and can identify unambiguously the GAD doublet. Moreover, titrations show a higher sensitivity of this method. Low titers in the RIA might be underestimated, in particular when indexes are used to compare different experiments (M.R.B., J.S. Petersen, A. Van Driel, C. Van Donselaar, G.J.B., T. Dyrberg, H.-J.A., unpublished observations).

We conclude that GAD antibodies are not prognostic for the rate of β-cell destruction or duration of the honeymoon phase in this group of young children. The observed qualitative and quantitative variability in GAD antibodies might explain the lack of correlation with clinical parameters. GAD antibodies are present in 70% of the patients at clinical onset, but in four out of 33 children (12%) GAD antibodies appeared weeks to months after diagnosis. If the observed fluctuations in GAD anti-

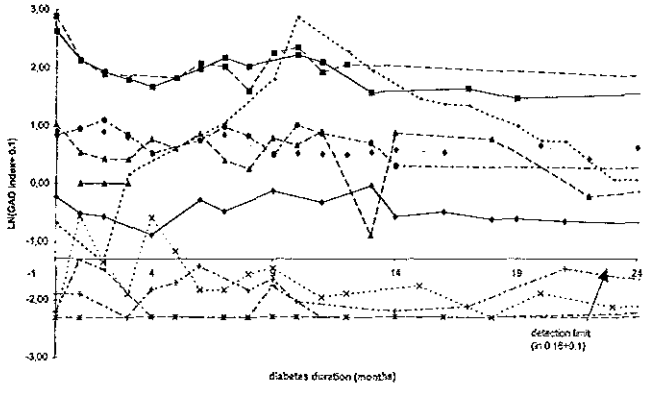

Figure 2—*The course of GAD indexes in 11 young patients during the initial 2 years of disease.*

Table 1—*Correlation coefficient of GAD indexes at onset with clinical parameters at onset and later in disease*

	Age-corrected C-peptide			GHb			Insulin dosage		
	Onset	3 months	6 months	Onset	3 months	6 months	Onset	3 months	6 months
All	−0.13			0.38			0.11		
	NS			$P = 0.037$			NS		
CSII		0.53	0.21		0.15	−0.10		0.16	−0.06
		NS	NS		NS	NS		NS	NS
Conventional		−0.03	−0.09		−0.53	0.32		−0.30	0.07
		NS	NS		NS	NS		NS	NS

bodies occur in the prediabetic phase as well, this has implications for diabetes prediction.

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References

1. Atkinson MA, Maclaren NK: The pathogenesis of insulin-dependent diabetes mellitus (Review). *N Engl J Med* 331:1428–1436, 1994
2. Aanstoot HJ, Sigurdsson E, Jaffe M, Shi Y, Christgau S, Grobbee D, Bruining GJ, Molenaar JL, Hofman A, Baekkeskov S: Value of antibodies to GAD65 combined with islet cell cytoplasmic antibodies for predicting IDDM in a childhood population. *Diabetologia* 37:917–924, 1994
3. Petersen JS, Dyrberg T, Karlsen AE, Molvig J, Michelsen B, Nerup J, Mandrup-Poulsen T: Glutamic acid decarboxylase (GAD65) autoantibodies in prediction of β-cell function and remission in recent-onset IDDM after cyclosporin treatment. The Canadian-European Randomized Control Trial Group. *Diabetes* 43:1291–1296, 1994
4. de Beaufort CE, Bruining GJ: Continuous subcutaneous insulin infusion in children. *Diabetic Med* 4:103–108, 1987
5. de Beaufort CE, Houtzagers CM, Bruining GJ, Aarsen RS, den Boer NC, Grose WF, van Strik R, de Visser JJ: Continuous subcutaneous insulin infusion (CSII) versus conventional injection therapy in newly diagnosed diabetic children: two-year follow-up of a randomized, prospective trial. *Diabet Med* 6:766–771, 1989
6. de Beaufort CE, den Boer NC, Bruining GJ, Eilers GA, van Strik R, Weterings T: Urinary C-peptide: a useful tool for evaluating the endogenous insulin reserve in cohort and longitudinal studies of diabetes in childhood. *Ann Clin Biochem* 25:552–559, 1988
7. Menard L, Dempsey M, Blanksteijn L, Alleyassine H, Wachs H, Soeldner J: Quantitative determination of glycosylated hemoglobin A1 by agar gel electrophoresis. *Clin Chem* 26:1598–1602, 1980
8. de Beaufort CE, Bruining GJ, Aarsen RS, de Boer NC, Grose WF: Does continuous subcutaneous insulin infusion (CSII) prolong the remission phase of insulin-dependent diabetes in children? Preliminary findings of a randomized prospective study. *Neth J Med* 1:53–54, 1985
9. Lernmark A, Kloppel G, Stenger D, Vathanaprida C, Falt K, Landin-Olsson M, Baskin DG, Palmer JP, Gown AM, Petersen JS, Li L, Edenvall H, Mauseth RS: Heterogeneity of islet pathology in two infants with recent onset diabetes mellitus (Review). *Virchows Arch* 425:631–640, 1995
10. Sorenson RL, Garry DG, Brelje TC: Structural and functional considerations of GABA in islets of Langerhans: β-cells and nerves (Review). *Diabetes* 40:1365–1374, 1991
11. Hyoty H, Hiltunen M, Knip M, Laakkonen M, Vahasalo P, Karjalainen J, Koskela P, Roivainen M, Leinikki P, Hovi T, Akerblom HK, the Childhood Diabetes in Finland (DiMe) Study Group: A prospective study of the role of coxsackie B and other enterovirus infections in the pathogenesis of IDDM. Childhood Diabetes in Finland (DiMe) Study Group. *Diabetes* 44:652–657, 1995
12. Kobayashi T, Itoh T, Kosaka K, Sato K, Tsuji K: Time course of islet cell antibodies and β-cell function in non-insulin-dependent stage of type I diabetes. *Diabetes* 36:510–517, 1987

Chapter 2.2

A longitudinal study of GAD- and IA2-antibodies in children with type-1 diabetes and their first-degree relatives

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2.2.1. Abstract

Antibodies to GAD and IA2 are increasingly recognized as sensitive and specific tools for the prediction of type-1 diabetes. Despite this, it is not clear when autoantibodies do first appear and if they are continuously present. In an 8 years prospective study of 75 children with long-standing diabetes and 252 first-degree relatives the persistence of GAD- and IA2-antibodies and fluctuations in antibody levels and status are evaluated.

Overall both GAD- and IA2-antibodies were decreasing in patients, but seroconversions to positive antibody levels occurred even 12 years after disease onset. In first-degree relatives GAD- and IA2-antibodies overall showed a slight decline, but GAD- and IA2-antibody levels were increased in early spring. In addition, we observed that seroconversions from negative to positive antibody levels may appear at any age and that such seroconversions may be transient.

These observations complicate autoantibody screening and warrant repeated sampling to improve sensitivity and specificity of diabetes prediction. In addition, the data suggest that environmental factors, possibly viral infections, may trigger ß-cell autoantibody formation in healthy first-degree relatives.

2.2.2. Introduction

GAD- and IA2-antibodies are increasingly recognized as sensitive and specific tools for the prediction of type-1 diabetes. Over 80% of patients with type-1 diabetes and individuals in the prediabetic phase are positive for one or both of these antibodies. It is not clear in what stage of prediabetes the antibodies appear, nor is it known if they are continuously present.

Studies on the appearance and fluctuations of antibodies in the prediabetic phase are scarce. Some authors suggest that ß-cell antibodies

generally develop early in life, before the age of two, but the follow-up in these studies is limited to 5 years [1, 2]. Others have demonstrated that antibodies may appear at any time in life, followed by spreading to additional islet antigens, a scenario that would severely complicate predictive autoantibody screening [3, 4]. Thus, it remains controversial at what stage of prediabetes autoantibodies appear and if they are present throughout the pre-clinical period.

In a study of 30 young children we demonstrated that GAD-antibody levels substantially fluctuated shortly after onset of diabetes, but are not correlated to c-peptide reserve or clinical parameters [5]. Others have described similar results, but in adults high antibody levels were correlated to a faster decline in ß-cell function, suggesting a correlation with the pathophysiology of type-1 diabetes [6-9].

The current study aims to evaluate: 1) the persistence of GAD- and IA2-antibodies at long disease duration; 2) if fluctuations in antibody levels or status are confined to recent onset of type-1 diabetes or may occur at longer disease duration as well; 3) if similar fluctuations occur in first-degree relatives. Therefore, the natural course of GAD- and IA2-antibodies during 8 years follow-up was evaluated in 75 children with type-1 diabetes, 150 of their parents and 102 siblings. In addition, we studied whether antibody levels were subject to seasonal fluctuations.

2.2.3. Methods

2.2.3.1. Population

Seventy-five patients of the diabetes clinic of the Sophia Children's hospital and 252 of their first-degree relatives participated in a study on prediction of type-1 diabetes. Serum was collected at four visits from March 1989 to June 1997, with time intervals as shown in table 2.2.1. Not all individuals participated at each visit. The mean duration of diabetes in the probands at the first visit in 1989 was 5.8 years and ranged from 0.6 to 18 years during the study. Their mean age at onset of

diabetes was 6.4 (range 1 month – 16.6 years). Other demographic data are described in table 2.2.1.

Informed consent was obtained from all participating individuals or their parents. The study was approved by the medical ethical committee of the Rotterdam University Hospital in 1989 in accordance with the declaration of Helsinki.

2.2.3.2. Laboratory tests

All sera were analyzed in triplicate for GAD- and IA2-antibodies by radiobinding assay (RBA) as described previously [10]. In vitro translated human recombinant GAD_{65} (a gift from Dr. T. Dyrberg, Gentofte, Denmark) and the intracellular domain of IA2 (AA 603-980; a gift from Dr. M. Christie, London, UK) were used as tracer. Precipitated radioactivity was counted in a microbeta plate reader (EG&G Wallac, Turku, Finland), correlated to negative and positive reference sera that were included in each 96 well plate and expressed as an antibody index [11]. The threshold for positivity was set at the 99.5th centile of 1403 schoolchildren for GAD-antibodies and 1085 schoolchildren for IA2-antibodies [10]. Sera with an antibody index between the 95th and 99th centile were considered dubious or ambiguous.

HLA-DR serotypes were determined as described by Giphart and co-workers [12].

2.2.3.3. Statistical analysis

Analyses of differences between groups were performed by the chi-square test for dichotomous variables and the Kruskal-Wallis H test for continuous variables using the statistical package SPSS for Windows (SPSS Inc, Chicago, Il. USA).

Table 2.2.1. Demographic data and GAD- and IA2-antibody test results of 75 families at 4 visits from 1989 to 1997

		1989	1991	1995	1997
Mean interval from first sample:			1.1 years	5.6 years	7.7 years
Patients	n	59	54	40	22
	age	12.2 (4.2–22.9)	12.81 (5.8–20.4)	16.5 (10.6–23.8)	18.0 (13.0-25.9)
	duration (yrs)	5.8 (3.0)	6.7 (2.9)	11.7 (3.1)	12.7 (2.5)
	GAD-index	0.0355 (59)	0.0213 (54)	0.0248 (40)	0.0501 (22)
	IA2-index	0.0036 (59)	-0.0012 (54)	0.0017 (40)	-0.0013 (11)
	n GADA+ (%)	13* (22%)	16* (29%)	13* (31%)	8* (36%)
	n GADA+/- (%)	19* (31%)	8* (14%)	7* (17%)	4* (18%)
	n IA2A+ (%)	18* (30%)	11* (20%)	3* (7%)	1 (9%)¶
	n IA2A +/- (%)	9 (15%)	7 (13%)	8 (22%)	1 (9%)
Mothers	n	71	62	43	26
	age	39.7 (30.1–50.8)	40.4 (31.3–49.2)	44.1 (36.1–53.7)	45.4 (38.3-55.8)
	GAD-index	0.0023	-0.0007	0.0035	-0.0057
	IA2-index	-0.0019	-0.0022	-0.0001	-0.0026
	n GADA+ (%)	1 (1%)	1 (2%)	1 (2%)	0
	n GADA+/- (%)	1 (1%)	2 (3%)	5 (11%)	4 (15%)
	n IA2A+ (%)	0	0	1 (2%)	0
	n IA2A +/- (%)	0	1 (2%)	0	0
Fathers	n	70	58	43	24
	age	41.9 (29.8–55.3)	42.4 (30.9–51.7)	45.5 (35.7–55.1)	47.5 (37.9-58.7)
	GAD-index	0.0024	-0.0010	-0.0002	-0.0015
	IA2-index	-0.0019	-0.0023	-0.0006	-0.0036
	n GADA+ (%)	2 (3%)	2 (4%)	2 (5%)	2 (8%)
	n GADA+/- (%)	1 (2%)	3 (5%)	3 (7%)	3 (13%)
	n IA2A+ (%)	1 (1%)	2 (3%)	0	0
	n IA2A +/- (%)	0	0	0	0
Sibs	n	89	71	66	39
	age	12.2 (1.9–23.5)	13.0 (4.16–23.9)	16.2 (4.5–28.4)	17.6 (6.6-30.6)
	GAD-index	0.0051	0.0004	0.0060	0.0005
	IA2-index	-0.0016	-0.0022	0.0002	-0.0021
	n GADA+ (%)	4 (4%)	2 (3%)	6 (9%)	2 (5%)
	n GADA+/- (%)	5 (6%)	4 (6%)	7 (11%)	3 (8%)
	n IA2A+ (%)	1 (1%)	0	1 (1%)	0
	n IA2A +/- (%)	0	1 (3%)	0	0

GADA: GAD-antibodies, IA2A: IA2-antibodies
GAD+: GAD-index > 99.5th centile of 1403 schoolchildren,
GAD+/-: GAD-index > 95th centile and < 99.5th centile of 1403 schoolchildren
IA2+: GAD-index > 99.5th centile of 1085 schoolchildren,
IA2+/-: IA2-index > 95th centile and < 99.5th centile of 1085 schoolchildren
* p<0.00 (patients versus relatives)
¶ p=0.014 (patients versus relatives)

To obtain a Gaussian distribution GAD-antibody indices and IA2-antibody indices were ln-transformed (lnGAD = (ln(GAD-index+0.08)) and lnIA2 = (ln(IA2-index+0.02)). The course of the antibody levels was analyzed using a repeated measurement model (SAS version 6.11: Proc Mixed [13]) with appropriate covariance structure. This model remains valid under less stringent assumptions about the randomness of the missing values than some popular methods (like 'Complete Cases' or 'Available Cases' analysis) and allows generally even selective missing values [14] A p-value < 0.05 was considered significant.

Table 2.2.2. Mean GAD- and IA2-indices calculated from the repeated measurements model for patients and relatives at four consecutive visits

visit	ln(GAD-index+0.08)	GAD-index	ln(IA2-index+0.02)	IA2-index
		Patients		
1	-1.55*	0.20	-3.11*	0.04
2	-1.61*	0.18	-3.42♣*	0.03
3	-1.70*	0.17	-3.53♣*	0.03
4	-1.92	0.14	-3.80♣	0.02
		Relatives		
1	-2.39*♥	0.08	-3.96*	0.02
2	-2.44♣	0.08	-3.97*	0.02
3	-2.37*	0.09	-3.88♣*♥	0.02
4	-2.53♣	0.07	-4.06♣	0.02

To facilitate interpretation the log values obtained in the mixed analysis are transformed to obtain antibody-indices.
♣p<0.05 compared to visit 1
♥p<0.05 compared to visit 2
*p<0.05 compared to visit 4

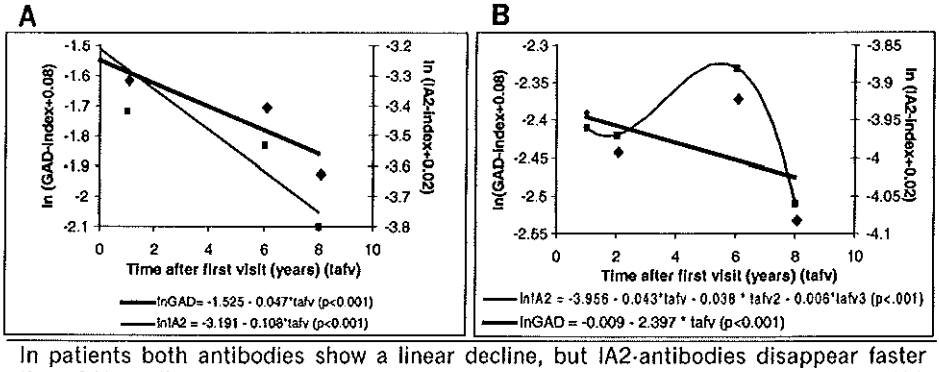
2.2.4. Results

Table 2.2.1. describes the observed GAD- and IA2-antibody levels and frequencies in patients and first-degree relatives at four visits. The frequency and median levels of GAD- and IA2-antibodies were significantly increased in the patients compared to the first-degree relatives in the samples collected at the first visit in 1989 ($p<0.001$). More patients than relatives had dubious GAD- and IA2-antibody levels ($p<0.001$).

2.2.4.1. Patients

The mean GAD- and IA2-antibody levels calculated from the repeated measurement model are shown in table 2.2.2. There is a significant decrease in lnGAD and lnIA2 levels during the study (P<0.01). Remarkably, the GAD-levels remain elevated compared to relatives throughout the study, while at visit 4 the mean IA2 levels were equal to the levels in relatives. From regression analysis in the repeated measurement model it was apparent that there was a more marked decline in GAD-antibody levels than in IA2-antibody levels (figure 2.2.1.).

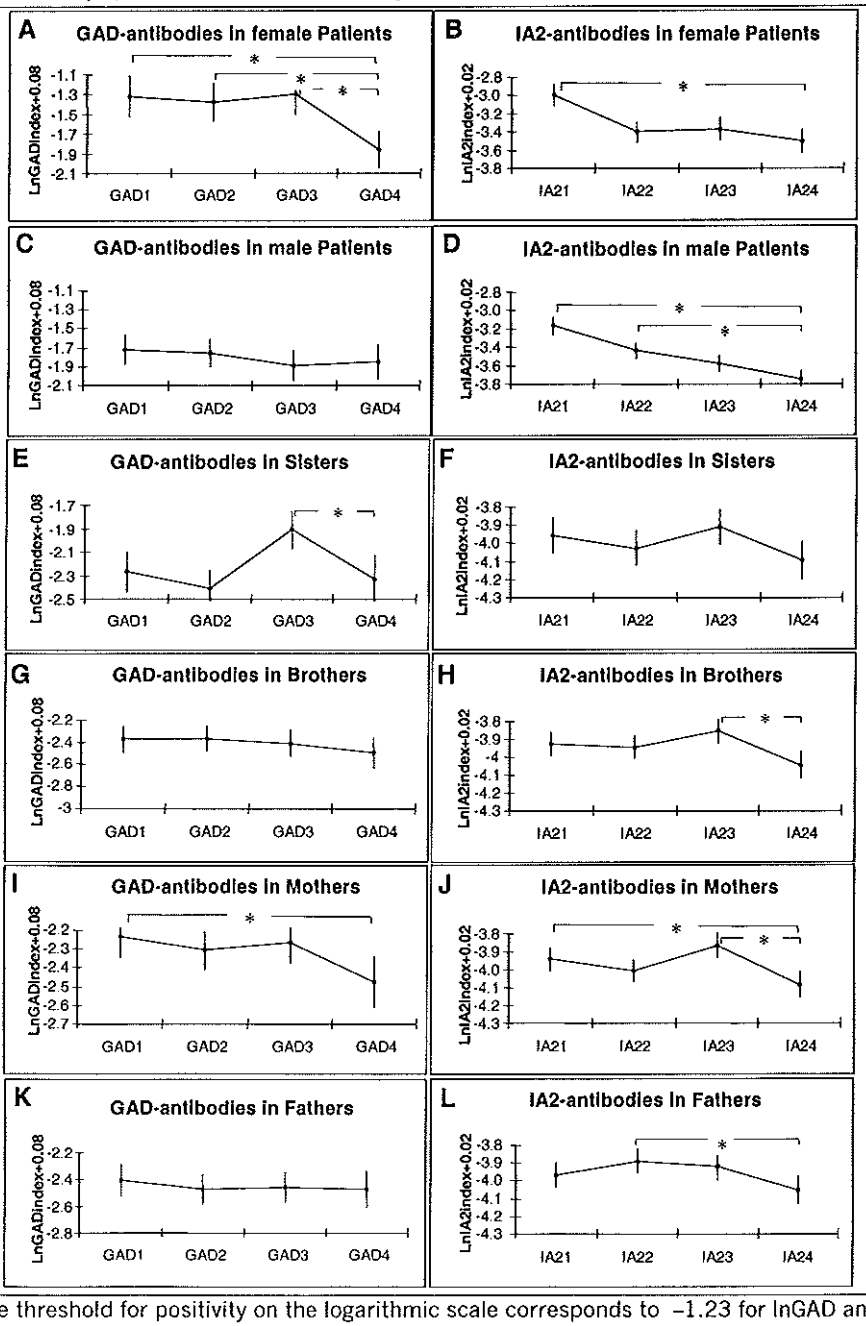
Figure 2.2.1. Regression analysis of lnGAD and lnIA2 in patients (A) and relatives (B) according to repeated measurement model.



In patients both antibodies show a linear decline, but IA2-antibodies disappear faster than GAD-antibodies. In first-degree relatives, lnGAD shows a linear decline and lnIA2 follows a polynomal pattern, antibody levels being significantly increased at the third visit.

Figure 2.2.2. panel A, B, C and D demonstrate the course of lnGAD and lnIA2 for male and female patients as calculated from the repeated measurement model. There was a significant decrease of GAD-antibodies over time in females but not in males. IA2-antibodies decreased significantly through time in both sexes. Figure 2.2.3. panel A and B illustrate the observed GAD- and IA2- antibody indices in all patients that had one or more positive or dubious samples during the study. IA2-antibody levels decreased throughout the study, whereas GAD-antibodies tended to remain more stable.

Figure 2.2.2. Course of lnGAD (A,C,E,G,I,K) and lnIA2 (B,D,F,H,J,L) in patients (A,B,C,D), sibs (E,F,G,H) and parents (I,J,K,L) during 8 years follow-up (calculated from the repeated measurements model).



The threshold for positivity on the logarithmic scale corresponds to –1.23 for lnGAD and to –2.5 for IA2. * $p<0.01$

These patterns are in agreement with the outcome of repeated measurement analysis, thus ratifying our previous observations using this model. Two patients were initially negative (GAD-levels <99.5th centile) and became positive for GAD-antibodies at later visits, at 2 and 12 years disease-duration. One patient seroconverted from positive (>99.5th centile) for IA2-antibodies at the first visit (IA2-index 0.24), at 4 years disease duration, to negative at the second visit (IA2-index -0.003) and positive again (IA2-index 0.13) at the third visit. This patient was dubious for GAD-antibodies throughout the study.

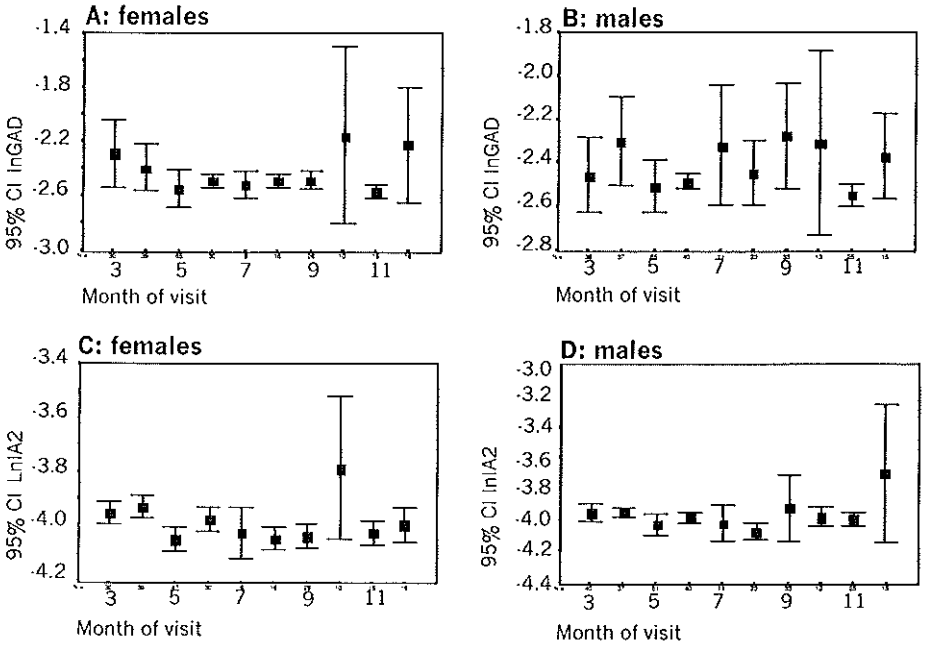
In order to analyze the effect of age of diagnosis on GAD- and IA2-antibody levels the probands were divided in three tertiles according to their age at diagnosis. There was no correlation between age of diagnosis and GAD- and IA2-antibody levels at the first visit (Spearman's correlation) and the antibody levels or frequencies at the first visit were not different between these age tertiles (data not shown). In addition, we divided the probands into risk-groups according to their HLA-phenotype: Children with high susceptibility (HLA-DR3/3, DR3/4, DR4/4), children with intermediate susceptibility (HLADR3/x or DR4/x (x representing any haplotype except DR3 or DR4)) and children with low susceptibility (DRx/x). There were no patients with DR2 phenotypes. The GAD- and IA2-antibody levels and frequencies at the first visit were not significantly different between those susceptibility groups (data not shown).

2.2.4.2. First-degree relatives

There was no significant difference in the observed antibody frequency or -level between mothers, fathers and sibs. LnGAD and InIA2 significantly decreased during the study when analyzing all first-degree relatives together in the repeated measurement model (figure 2.2.2.). This decrease was not reflected by the index values and thus this observation has no clinical significance (table 2.2.2.).

Strikingly, there was a significant increase in lnGAD and lnIA2 at the third visit. This increase was more marked in female than male relatives and independent from assay conditions (figures 2.2.1. and 2.2.2.). Female patients, but not male patients, followed a similar pattern. Visit 3 mostly occurred during early spring, while visit 1, 2 and 4 took place in a widespread period from March to December. Therefore we analyzed whether the increased antibody levels at visit 3 could be due to seasonal variations. Figure 2.2.4. demonstrates the observed levels of both lnGAD and lnIA2 in different months (March to December). Antibody levels tended to be higher in early spring, especially in female relatives. Using the repeated measurement analysis and correcting for the visit number the increase was significant in March and April compared to all other months (data not shown).

Figure 2.2.4. Seasonal fluctuations of GAD- (A & B) and IA2-antibodies (C & D) in male (B & D) and female (A & C) relatives.



Antibody levels are expressed as lnGAD and lnIA2. Bars represent the 95% confidence interval (CI) of lnGAD and lnIA2. Fluctuations occur within the normal ranges.

During the entire study period seroconversions from previously negative to dubious or positive GAD-antibody status were observed in 30 first-degree relatives (12%) (figure 2.2.3., table 2.2.3.). At the second visit two first-degree relatives converted from seronegative to positive or ambiguous GAD-antibody levels, accompanied by a conversion in IA2-antibodies. No follow-up samples were available of these individuals and they did not develop diabetes during follow-up.

At the third visit 16 individuals seroconverted for GAD-antibodies from negative to dubious antibody levels. Of those, seven experienced a transient increase, of the remaining nine individuals there were no further samples available. Two individuals converted from negative to positive, in one of these the increase was transient, while the other remained positive in the follow-up sample (Table 2.2.3.). None of the GAD-antibody seroconversions in the third sample were accompanied by seroconversions in IA2-antibodies. Strikingly, 6 of 18 seroconversions occurred within two families, suggesting that there was a common agent causing the transient increase in GAD-antibody levels. These two families visited the Sophia Children's Hospital at separate occasions. At visit 4 we observed similar clustering of fluctuations: four out of nine seroconversions (from negative to dubious) occurred within two families.

Seroconversions for IA2-antibodies in first-degree relatives were uncommon: only three individuals that were negative at the first visit converted to positive in a later sample. No follow-up samples were available of these individuals. Two other relatives were positive for IA2-antibodies in their initial sample in 1989, but turned negative during follow-up (figure 2.2.3., table 2.2.3.).

Of 252 relatives three (1.2%) developed type-1 diabetes between the second and the third visit. Two conversions occurred within one family. The antibody status of these individuals at all visits are shown in boldface in table 2.2.3. Fifteen other first-degree relatives (6%) were positive for any antibody in at least one serum sample, but did not develop diabetes

during follow-up (table 2.2.3.). Our observations demonstrate that seroconversions may occur at any age and individuals may be transiently positive for GAD- or IA2-antibodies.

Table 2.2.3. First-degree relatives with at least one positive sample for GAD- or IA2-antibodies during the study.

relation to proband	age at 1st visit	Antibodies against	visit 1	visit 2	visit 3	visit 4
mother*	**43**	**GAD**	**n.t.**	**+**	**+**	**n.t.**
DR3/4		**IA2**	**n.t.**	**+/-**	**+**	**n.t.**
brother*	**15**	**GAD**	**+**	**+**	**+**	**n.t.**
DR3/4		**IA2**	**-**	**-**	**-**	**n.t.**
mother	**31**	**GAD**	**-**	**n.t.**	**-**	**-**
DR3/7		**IA2**	**-**	**n.t.**	**-**	**-**
sibling	23	GAD	+	n.t	+	n.t
DR4/4		IA2	+	n.t	-	n.t
sibling	22	GAD	-	n.t	+	n.t.
DR3/7		IA2	-	n.t.	-	n.t.
sibling	11	GAD	+	n.t.	n.t.	n.t.
DR3/9		IA2	-	n.t.	n.t.	n.t.
sibling	10	GAD	-	-	+	-
DR1/4		IA2	-	-	-	n.t.
sibling	15	GAD	-	+	n.t.	n.t.
n.t.		IA2	-	+/-	n.t.	n.t.
sibling	15	GAD	+	+	+/-	n.t.
DR5/W6		IA2	+	+	+/-	n.t.
sibling	11	GAD	-	-	+	+
DR3/3		IA2	-	-	-	-
sibling	20	GAD	+	n.t.	+	+
n.t.		IA2	-	n.t.	-	-
father	33	GAD	-	+/-	n.t.	n.t.
DR4/6		IA2	-	+	n.t.	n.t.
father	36	GAD	+	+/-	n.t.	n.t.
DR2/4		IA2	-	-	n.t.	n.t.
father	39	GAD	+/-	+	+	+
DR3/3		IA2	-	-	-	-
father	41	GAD	n.t.	+	+	+
DR3/4		IA2	n.t.	-	-	-
father	45	GAD	-	n.t.	-	n.t.
DR3/W8		IA2	+	n.t.	-	n.t.
father**	48	GAD	+	+/-	n.t.	n.t.
DR3/W10		IA2	-	-	n.t	n.t
mother**	47	GAD	+	-	n.t.	n.t.
DR1/3		IA2	-	-	n.t.	n.t.

Individuals printed in boldface developed diabetes between visit 2 and 3.
** 2 individuals from one family.

To analyze whether antibody levels are correlated to age, the sibs were stratified to three age tertiles according to their age at the first visit. The

GAD- or IA2-antibody frequencies at the first visit did not differ between these tertiles and there was no correlation between age and antibody levels (Spearman's correlation test). Similar to the patients, the relatives were divided into HLA-susceptibility classes. The antibody levels or frequencies at the first visit were similar in all HLA-susceptibility classes, either analyzed for all relatives together or for parents and sibs separately. Seroconversions were not confined to a special susceptibility group.

2.2.5. Discussion

The current study aimes to evaluate fluctuations in antibody levels and status in patients with type1 diabetes and their first-degree relatives. Islet cell antibodies (ICA) were not included in this study since most studies nowadays have proved that prediction using GAD- and IA2-antibodies can replace ICA screening. In addition, quantification of ICA titers requires high serum volumes and a one-step dilution artefact will significantly hamper the analysis.

2.2.5.1. Patients

In the current study we demonstrate that GAD- and IA2-antibodies may remain present in the circulation of patients with type-1 diabetes long after diagnosis. However, there is a general decreasing trend for GAD- and IA2-antibodies in patients and IA2-antibody levels tend to decrease more rapidly than GAD-antibodies. Similar observations came from other studies [6, 15, 16].

Although the current study was not specifically designed to detect frequent fluctuations in antibody levels and the sampling frequency was not as frequent as in our previous study [5], we detected two patients that converted from negative to positive for GAD-antibodies after disease onset. In addition, one patient showed striking conversions of IA2-antibodies from negative to positive and back. GAD- and IA2-antibody

seroconversions did not occur simultaneously within one patient, indicating that the fluctuations are due to antigen specific events. Seroconversions occurred between two and 12 years of disease duration, suggesting that ß-cell specific immune activation may occur long after onset of diabetes when supposedly all ß-cells have been destroyed. A sudden revival of ß-cell regeneration up to 12 years after disease onset is likely to occur. Therefore, it is tempting to conclude that that external sources (viral, bacterial or nutritional antigens mimicking GAD_{65} or IA2) or a sudden release of GAD_{65} or IA2 from other tissues (due to trauma or infections) cause the ß-cell specific immune-activation. We previously demonstrated that in non-diabetic children destruction of tissues that express GAD_{65} does not lead to antibody formation [17]. However, it is conceivable that in patients with diabetes the genetic background or the immunological memory may facilitate such a reaction.

2.2.5.2. First-degree relatives

Three out of 252 relatives (1.2%) converted to diabetes during 8 years follow-up. Two converters, a mother and a brother of the proband, came from one family. Both were positive for GAD-antibodies prior to diabetes onset. The brother remained negative for IA2-antibodies throughout the study, while the mother of the same proband showed increasing IA2-antibody levels and was positive for IA2-antibodies after clinical manifestation. The proband in this family was positive for GAD-antibodies and negative for IA2-antibodies throughout the study. All patients in this family were HLA-DR3/4. Thus, antibody patterns in multiplex families are not necessarily the same in all patients, although they share HLA-phenotypes, suggesting that the pathogenesis in individuals with a similar genetic background may be heterogeneous. The third individual (a mother of a proband) who developed diabetes at follow-up was negative for all antibodies tested, including islet cell antibodies and antibodies against GLIMA38 [18] The HLA type of this woman was HLA-DR3/7. Her

diabetic son, who HLA type was HLA-DR3/4, was negative for all antibodies tested as well (first visit at 6 years disease duration).

Overall antibody levels were stable in first-degree relatives. However, fluctuations in antibody levels, and occasionally status, occurred in 12% of the relatives. During the study six individuals became positive for either GAD- or IA2-antibodies after a previous negative or dubious sample at ages ranging from 16 to 34 years of age. Of these seroconverters two remained positive in follow-up samples, one was transiently positive and of the other three no follow-up samples were available. At a recurrence rate of 3% among first-degree relatives four more cases of diabetes are to be expected in this cohort. It is likely that anyone among those who seroconverted will develop diabetes in the future. Thus, in contrast to a large number of studies that favor the early initiation of ß-cell autoimmunity and consequent development of ß-cell antibodies early in life [1, 19, 20], we demonstrated that antibodies may appear at any age. These observations are ratified by other studies [3, 4, 21, 22] and suggest that at least a subgroup of type-1 diabetes is not solely caused by perinatal events.

Two relatives were transiently positive for GAD-antibodies. Currently, it is not clear if these individuals will develop diabetes in the future, but this observation might have serious consequences for the design of antibody screening strategies.

Most seroconversions occurred early in spring and seroconversions were clustered within families. In addition, we demonstrated that in first-degree relatives antibody levels tend to be higher early in spring than during summer months. Thus, there is clustering in time and space of GAD- and IA2-antibody fluctuations, suggestive for environmental factors occurring in a seasonal pattern to play a role. The seasonal occurrence of enteroviral infections coincides with the antibody pattern and several studies have implicated a role for enteroviral infections in the pathophysiology of type-1 diabetes [23-27]. A structural homology between coxsackievirus-proteins

and GAD_{65} has been reported [28-30] and Hiltunen and colleagues described that coxsackie virus infections may be accompanied by transient increases in islet cell antibodies [31]. From the current study it is not clear whether the increased antibody levels and transient positivity are associated with later development of type-1 diabetes. More detailed studies of these (temporarily) antibody positive individuals, including viral load and longer follow-up may yield additional information on the pathogenesis of type-1 diabetes.

2.2.5.3. Conclusion

We conclude that there is a general decreasing trend of GAD- and IA2-antibodies in patients with type-1 diabetes, but substantial fluctuations may occur even long after disease onset. Such fluctuations are likely to be due to an external antigen source. In relatives fluctuations in antibody levels occurred in a seasonal pattern and were clustered within families, suggesting that viral infections play a role. Furthermore, this study demonstrates that healthy first-degree relatives may be transiently positive for GAD-antibodies and seroconversions may occur at any age, thus complicating diabetes prediction strategies and warranting repeated sampling.

Acknowledgements

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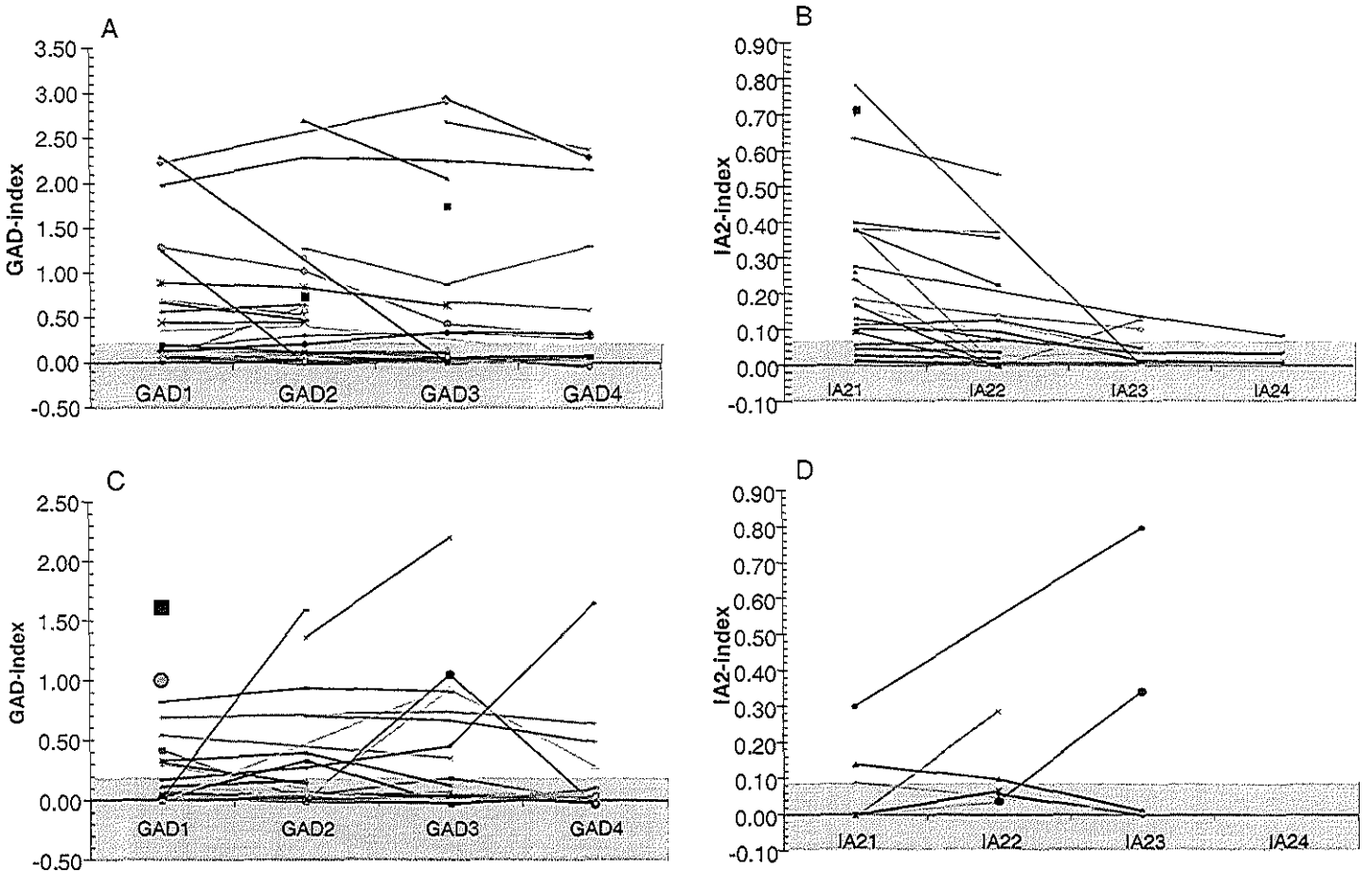
References:

1. Ziegler AG, Hillebrand B, Rabl W, Mayrhofer M, Hummel M, Mollenhauer U, et al. On the appearance of islet associated autoimmunity in offspring of diabetic mothers: a prospective study from birth. *Diabetologia* 1993;36(5):402-8.
2. Roll U, Christie MR, Fuchtenbusch M, Payton MA, Hawkes CJ, Ziegler AG. Perinatal autoimmunity in offspring of diabetic parents: the german multicenter BABY-DIAB study: detection of humoral immune responses to islet antigens in early childhood. *Diabetes* 1996;45(7):967-973.
3. Yu L, Rewers M, Gianani R, Kawasaki E, Zhang Y, Verge C, et al. Antiislet autoantibodies usually develop sequentially rather than simultaneously. *J Clin Endocrinol Metab* 1996;81(12):4264-7.
4. Bonifacio E, Bingley PJ, Shattock M, Dean BM, Dunger D, Gale EA, et al. Quantification of islet-cell antibodies and prediction of insulin- dependent diabetes. *Lancet* 1990;335(8682):147-9.
5. Batstra MR, Pina M, Quan J, Mulder P, de Beaufort CE, Bruining GJ, et al. Fluctuations in GAD65 antibodies after clinical diagnosis of IDDM in young children. *Diabetes Care* 1997;20(4):642-4.
6. Savola K, Sabbah E, Kulmala P, Vahasalo P, Ilonen J, Knip M. Autoantibodies associated with Type I diabetes mellitus persist after diagnosis in children. *Diabetologia* 1998;41(11):1293-7.
7. Komulainen J, Knip M, Lounamaa R, Vahasalo P, Karjalainen J, Sabbah E, et al. Poor beta-cell function after the clinical manifestation of type 1 diabetes in children initially positive for islet cell specific autoantibodies. The Childhood Diabetes in Finland Study Group. *Diabet Med* 1997;14(7):532-7.
8. Hoeldtke RD, Bryner KD, Horvath GG, Byerly MR, Hobbs GR, Marcovina SM, et al. Antibodies to GAD and glycemic control in recent-onset IDDM. *Diabetes Care* 1997;20(12):1900-3.
9. Petersen JS, Dyrberg T, Karlsen AE, Molvig J, Michelsen B, Nerup J, et al. Glutamic acid decarboxylase (GAD65) autoantibodies in prediction of beta-cell function and remission in recent-onset IDDM after cyclosporin treatment. The Canadian-European Randomized Control Trial Group. *Diabetes* 1994;43(11):1291-6.
10. Batstra MR, Petersen J, Verwest A, Man de S, Molenaar J, Grobbee D, et al. Can we predict diabetes in schoolchildren? Threshold definition in GAD- and IA2 antibody assays. *Submitted* 1999.
11. Petersen JS, Hejnaes KR, Moody A, Karlsen AE, Marshall MO, Hoier-Madsen M, et al. Detection of GAD65 antibodies in diabetes and other autoimmune diseases using a simple radioligand assay. *Diabetes* 1994;43(3):459-67.
12. Giphart MJ, Roep BO, Drabbels J, D'Amaro J, Bruining GJ, Abdulkadir J, et al. Relative contribution of HLA-DQA and -DQB alleles to predisposition to insulin-dependent diabetes mellitus. *Hum Immunol* 1992;34(2):142-6.
13. Little R, Rubin D. *Statistical analysis with missing data*. New York, NY, USA: John Wiley & Sons, 1987.
14. Changes and Enhancements through release 6.11 [program]. Cary, NC, USA: SAS Institute Inc., 1996.
15. Christie MR, Daneman D, Champagne P, Delovitch TL. Persistence of serum antibodies to 64,000-Mr islet cell protein after onset of type I diabetes. *Diabetes* 1990;39(6):653-6.
16. Roll U, Nuber A, Schroder A, Gerlach E, Janka H, Ziegler A. No association of antibodies to glutamic acid decarboxylase and diabetic complications in patients with IDDM. *Diabetes Care*. 1995;18:210-5.
17. Batstra MR, van Driel A, Petersen JS, van Donselaar C, van Tol MJ, Bruining GJ, et al. GAD-antibodies in screening for autoimmune diabetes - influence of age, sex and other diseases on specificity and threshold levels. *Submitted*. 1999.

18. Aanstoot HJ, Kang SM, Kim J, Lindsay LA, Roll U, Knip M, et al. Identification and characterization of glima 38, a glycosylated islet cell membrane antigen, which together with GAD65 and IA2 marks the early phases of autoimmune response in type 1 diabetes. *J Clin Invest* 1996;97(12):2772-83.
19. Bingley PJ. Interactions of age, islet cell antibodies, insulin autoantibodies, and first-phase insulin response in predicting risk of progression to IDDM in ICA+ relatives: the ICARUS data set. Islet Cell Antibody Register Users Study. *Diabetes* 1996;45(12):1720-8.
20. Martikainen A, Saukkonen T, Kulmala PK, Reijonen H, Ilonen J, Teramo K, et al. Disease-associated antibodies in offspring of mothers with IDDM. *Diabetes* 1996;45(12):1706-10.
21. Roll U, Christie MR, Standl E, Ziegler AG. Associations of anti-GAD antibodies with islet cell antibodies and insulin autoantibodies in first-degree relatives of type I diabetic patients. *Diabetes* 1994;43(1):154-60.
22. Riley WJ, Maclaren NK, Krischer J, Spillar RP, Silverstein JH, Schatz DA, et al. A prospective study of the development of diabetes in relatives of patients with insulin-dependent diabetes. *N Engl J Med* 1990;323(17):1167-72.
23. Yoon JW. The role of viruses and environmental factors in the induction of diabetes. *Curr Top Microbiol Immunol* 1991;164:95-123.
24. Foulis AK, McGill M, Farquharson MA, Hilton DA. A search for evidence of viral infection in pancreases of newly diagnosed patients with IDDM. *Diabetologia* 1997;40(1):53-61.
25. Andreoletti L, Hober D, Hober-Vandenberghe C, Belaich S, Vantyghem MC, Lefebvre J, et al. Detection of coxsackie B virus RNA sequences in whole blood samples from adult patients at the onset of type I diabetes mellitus. *J Med Virol* 1997;52(2):121-7.
26. Dahlquist G, Frisk G, Ivarsson SA, Svanberg L, Forsgren M, Diderholm H. Indications that maternal coxsackie B virus infection during pregnancy is a risk factor for childhood-onset IDDM. *Diabetologia* 1995;38(11):1371-3.
27. Hyoty H, Hiltunen M, Knip M, Laakkonen M, Vahasalo P, Karjalainen J, et al. A prospective study of the role of coxsackie B and other enterovirus infections in the pathogenesis of IDDM. Childhood Diabetes in Finland (DiMe) Study Group. *Diabetes* 1995;44(6):652-7.
28. Atkinson MA, Bowman MA, Campbell L, Darrow BL, Kaufman DL, Maclaren NK. Cellular immunity to a determinant common to glutamate decarboxylase and coxsackie virus in insulin-dependent diabetes [see comments]. *J Clin Invest* 1994;94(5):2125-9.
29. Lonnrot M, Hyoty H, Knip M, Roivainen M, Kulmala P, Leinikki P, et al. Antibody cross-reactivity induced by the homologous regions in glutamic acid decarboxylase (GAD65) and 2C protein of coxsackievirus B4. Childhood Diabetes in Finland Study Group. *Clin Exp Immunol* 1996;104(3):398-405.
30. Vreugdenhil GR, Geluk A, Ottenhoff TH, Melchers WJ, Roep BO, Galama JM. Molecular mimicry in diabetes mellitus: the homologous domain in coxsackie B virus protein 2C and islet autoantigen GAD65 is highly conserved in the coxsackie B-like enteroviruses and binds to the diabetes associated HLA-DR3 molecule. *Diabetologia* 1998;41(1):40-6.
31. Hiltunen M, Hyoty H, Knip M, Ilonen J, Reijonen H, Vahasalo P, et al. Islet cell antibody seroconversion in children is temporally associated with enterovirus infections. Childhood Diabetes in Finland (DiMe) Study Group. *J Infect Dis* 1997;175(3):554-60.


A
GAD-index
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
-0.50
GAD1
GAD2
GAD3
GAD4
B
IA2-index
0.90
0.80
0.70
0.60
0.50
0.40
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0.20
0.10
0.00
-0.10
IA21
IA22
IA23
IA24
C
GAD-index
2.50
2.00
1.50
1.00
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0.00
-0.50
GAD1
GAD2
GAD3
GAD4
D
IA2-index
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Chapter 3

Prediction in the general population

Chapter 3.1

Antibody Screening in a Population of Children

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Special Section: Childhood Diabetes – New Insights into Pathogenesis, Treatment and Prevention

Antibody Screening in a Population of Children

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The first large-scale (secondary) intervention trials have been initiated in first-degree family members of patients with insulin-dependent diabetes mellitus (IDDM). Within a few years, data from these studies may suggest that intervention is possible, thereby opening similar approaches in the general population. However, before large-scale intervention studies can be initiated, several problems need to be solved. One of these problems is the lack of knowledge on the natural course of β-cell autoimmunity. This review analyses this and other issues related to population-based prediction for IDDM. At present, no long-term follow-up studies are available in large-sized populations, but data show that prediction in the general population is both technically feasible and likely to have sufficient power to be useful in prevention trials. More data need to be generated, not only to determine which markers are most likely to give good prediction but also to obtain knowledge on the natural course, psychosocial impact and cost-effectiveness of screening.

Key words: autoantibodies; autoimmunity; glutamic acid decarboxylase; IA-2; insulin-dependent diabetes mellitus; islet cell antibodies; prediabetes; prediction.

(Annals of Medicine 29: 453–460, 1997)

Introduction

Currently, several trials on prevention of insulin-dependent diabetes mellitus (IDDM) are in progress. Most of these trials focus on prevention in first-degree relatives of patients with IDDM. However, this accounts for a maximum 10% of novel cases of diabetes because most new cases occur in individuals without an affected first-degree relative. A significant impact on prevention of the incidence of IDDM will only be achieved once individuals from the general population with an increased risk for diabetes can be identified and acceptable intervention has become available. Therefore, IDDM prediction strategies that are sensitive and technically applicable to the general population are a pre-requisite for the coming years, while studies on this issue are urgently required now. Prediction of IDDM has been studied extensively in first-degree relatives of IDDM patients. Using autoantibody markers IDDM can be predicted with a sensitivity of over 85% in a family-based population (1, 2). Although one group reported similar predictive values for autoantibodies in the general population and a family-based population (3), it is generally assumed that the predictive value of antibody screening in the general population is 5–10 times lower than among first-degree relatives (4–6).

The first events in the pathogenesis of IDDM are likely to occur early in life (7–9), suggesting that intervention might be effective only when initiated in childhood. It is clear that any intervention applied in children requires thorough evaluation, and also simple and reliable prediction methods applicable to existing baby and child health welfare systems. This review analyses the present status of prediction in the general population and describes which markers can be applied, when and how often a population should be screened and what the prospects of screening in the general population are.

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Genetic or Immunological Markers?

IDDM is characterized by a long preclinical phase. During this phase, which may last from months to years, a gradual decline of the β-cell mass occurs owing to

continuous or intermittent attacks of the immune system on the β cells. This prediabetic period offers unique opportunities for both disease prediction and prevention.

IDDM prediction can be performed using genetic and immunological markers. Among the genetic markers, most is known about the HLA genes, DR and DQ genes being the most informative markers for genetic susceptibility. Up to 90% of IDDM patients carry susceptible HLA-DR and DQ types (10–12), which is consistent with a high sensitivity of HLA screening in IDDM. However, up to 60% of healthy individuals may carry susceptible HLA-DR and DQ haplotypes (10), resulting in low specificity of HLA screening.

The genome-wide screening for IDDM has resulted in the identification of several other genes involved in disease susceptibility (13, 14). It is conceivable that not only immune system-related genes but also genes related to β-cell development are involved. An example of this nonimmunological genetic basis comes from studies on nonobese diabetic (NOD) mice. NOD mice feature abnormal islets, probably the result of aberrant β-cell development. These large islets of Langerhans show different distributions of insulin expression and may have a capillary bed differing from normal-sized islets (15). It is likely that such aberrant developmental patterns are caused by genetic defects and may play a role in the pathogenesis of diabetes in NOD mice. The substantial decrease of susceptibility to autoimmune diabetes in NOD mice after a back-cross with other mouse strains (16) suggests that various diabetes susceptibility genes interact in a multiplicative manner. Such a genomic predisposition will lead to clinical manifestation of diabetes early in life. Individuals with fewer susceptibility genes are likely to need a stronger environmental trigger, consequently diabetes may occur at any age after encountering this trigger. Thus, the identification of additional IDDM susceptibility loci may enable the development of more specific genetic screening techniques for IDDM, especially for those developing IDDM early in life, eventually leading to primary prediction and prevention (before initiation of the disease process) of IDDM in the general population. Current genetic prediction is hampered by low specificity. It is, however, possible (and already in use; see other articles in this issue) to apply genetic susceptibility markers as the first selection ('sieve') of those at risk. These studies, testing all new-borns for the high- and intermediate-risk HLA haplotypes, select 10–50% (depending on their specificity) of the population, in which 70–98% of the future cases will be represented. While at present primary prevention based on genetic susceptibility is limited to the Finnish milk intervention study, follow-up with immunological markers will further identify those who would require secondary intervention.

Immunological markers have better specificity as they mark the ongoing disease process. At clinical onset 80–85% of the newly diagnosed patients with IDDM are positive for one or more β-cell autoantibodies (17–20), indicating high sensitivity of screening. The specificity of autoantibody screening in family-based populations is high, with a predictive power approaching 100% for young siblings with high antibody titres (19). Calculations of predictive power and assay sensitivity and specificity are often based on antibody frequencies in newly diagnosed patients and extrapolations of frequencies in the general population. However, it is at present not unlikely that the antibody profile during pre-diabetes differs from clinical diagnosis. Therefore follow-up studies of large groups of individuals at risk are a prerequisite to determine true predictive values.

The specificity of autoantibody screening in the general population is strongly dependent on their background frequencies. Autoantibody assays are now applicable to large populations and the first studies of their background frequencies in the general population are available (4, 5, 21–25). However, frequencies from less than 0.3% up to 5% are reported. This might be due to the different populations as well as to the methods used. There is a lack of long-term studies that have incorporated progression to IDDM. Future population-based studies should therefore incorporate genetic markers, multiple autoantibodies and long-term follow-up to IDDM.

Which Immunological Markers Are Available?

The ongoing intervention studies such as the Diabetes Prevention Trial (DPT1) and the European Nicotinamide Diabetes Prevention Trial (ENDIT) use the islet cell antibody (ICA) titre as well as additional markers in first-degree relatives as inclusion criteria. ICA have been used in population studies. In a group ($n=2805$) of healthy school children we found 0.3% (8 children) to be positive for complement fixing ICA (26). This includes four of seven children who developed diabetes during an 11.5-year follow-up. Glutamic acid decarboxylase (GAD) antibodies were more specific and sensitive: one additional future case of diabetes and only two of four ICA-positive children who did not develop IDDM were positive for GAD antibodies. In this population only one of seven future cases was insulin autoantibody (IAA) positive (21). Hagopian et al. reported an ICA frequency of 4% in a population of healthy children, which is at least 10 times higher than the expected IDDM prevalence in this population (6). Consequently the predictive value of ICA in the general population is low. Similar frequencies and predictive values were found for GAD antibodies and IAA. A combination of GAD antibodies and ICA had the best predictive value (6). Despite ICA standardization workshops organized by the Immunology of Diabetes Society and the development of standard Juvenile Diabetes Foundation Units (JDFU), ICA determinations remain technically difficult and strongly dependent on the pancreas substrate (27–29). In addition, for quantification of ICA relatively large serum volumes are required, reducing the applicability of ICA screening to large cohorts. In conclusion, ICA do not seem to be the best markers and technical limitations prevent their application in population-based

prediction programmes. Thus, similar to what has been proposed for first-degree relative-based studies, antibody analysis using molecularly defined antigens are required for population-based prediction (30–32).

Insulin was the first molecularly characterized autoantigen described in IDDM (33) and attracts attention as it is the only (at least after birth) β-cell-specific protein. While insulin may play a role in the pathogenesis of diabetes, the IAA remain a controversial issue. The frequency of IAA in IDDM, pre-IDDM and the general population has been reported to be inversely correlated to age (6, 7, 34–37), thus limiting the use of IAA in IDDM prediction. Technical aspects of the assay further diminish the applicability of IAA for screening of large cohorts: large serum volumes are required for IAA determination and the assay requires long incubation times owing to an apparent extremely low affinity of the IAA to insulin. It is impossible in native conditions to immunoprecipitate insulin by IAA-positive sera, raising the question of whether proteins other than insulin might be involved in the binding of the autoantibodies to their target.

Molecularly defined antigens that can be immunoprecipitated by sera of newly diagnosed IDDM patients and prediabetic individuals include GAD (38), IA-2 (39) and IA-2β (40) and GLIMA38 (18). The precipitating antibodies are directed against conformational epitopes (18, 38, 41), thereby requiring assays in which the target protein has the correct three-dimensional form.

The formation of IA-2β antibodies is secondary to an IA-2 autoimmune response, limiting the use of a separate IA-2β assay (42). IA-2 and GAD antibodies can be tested in a combined assay, enabling high throughput screening with an optimal predictive value. In family-based populations a high predictive value can be achieved by combining two or more antibody assays (19, 43–45). However, the best combination of antibodies for highly sensitive and specific screening in the general population remains to be established. Further research into the molecular identity of the GLIMA38 protein might eventually enable the combination of assays for three conformational β-cell autoantibodies.

When Should Screening Commence?

At present, sufficient data are lacking to determine when antibody testing should commence and at which frequency. This is due mainly to lack of knowledge on the natural course of autoimmunity in IDDM. It is not unlikely that more than one mechanism can result in the destruction of β cells. Two hypotheses (β-cell destruction starts early in life vs. β-cell destruction can start at any moment in life) represent different views, but would require two different screening strategies. If we presume that the process leading to clinical diabetes is caused by a β-cell attack very early in life, with concomitant appearance of autoantibodies that remain detectable until clinical onset of the disease, one single screening could suffice. This hypothesis of prediabetes is based on a model in which susceptible genes plus 'events' in the prenatal, neonatal and perinatal phase are major initiators of the disease process. Alternatively, it is conceivable that genes are less important and environmental factors (probably the sum of more than one environmental factor) later in life are required to initiate autoimmunity. In this model autoantibodies may appear for the first time at any time in life and fluctuations in immune activation and antibody titres are likely over time. Repeated analyses would be required to achieve high sensitivity of antibody screening. As suggested, it is not unlikely that both mechanisms coexist. It is of paramount importance to establish the natural course of β-cell autoimmunity in order to learn when screening should start and how frequently screening is required to achieve acceptable prediction. The recently initiated German Multicenter BABY-DIAB study and the Diabetes Autoimmunity Study in the Young (DAISY) provide such data in children with a positive family history (34, 46, 47). However, at present follow-up in both studies is still limited and longer follow-up and extensive studies in large newborn cohorts in other countries are still required.

In the BABY-DIAB study β-cell autoantibodies in cord blood of offspring of diabetic parents were strongly correlated to the presence of ICA, IAA, GAD and IA-2 autoantibodies in the maternal circulation and antibodies disappeared in most newborns during follow-up, indicating placental transmission (34, 48). Some children remained positive for IAA in a blood sample taken nine months postpartum. The studies of Martikainen et al. in Finland describe the appearance of autoantibodies at 6 months in a child that developed IDDM at 14 months (49). In the BABY-DIAB study antibodies appeared before the age of 2 in all children of diabetic parents, but the maximum follow-up duration is limited to 5 years ($n=37$) (7, 34). Thus, detection of β-cell autoantibodies after 9 months of age indicates the development of a β-cell-directed autoimmune response in these children and might be predictive for future diabetes. This supports the hypothesis that early life events are indeed involved in initiating the disease.

β-cell autoantibodies in healthy first-degree relatives of IDDM patients can be detected as early as 2 years postpartum (2). In the DAISY study the age of children being positive for any antibody (GAD, IA-2 or IAA) ranged from 0.7 to 7.1 years. No baseline data of these children are available, so that the time point of seroconversion remains obscure. The second hypothesis suggests that autoantibodies can appear at any moment in life and our own data support this possibility of late seroconversion. In first-degree relatives of 83 families with a child with IDDM one father became positive for GAD antibodies at the age of 46. In another family a sibling became positive for ICA at the age of 17, while two samples 2 and 4 years previously were negative (samples were tested in similar assay on three occasions). Neither of the individuals developed diabetes during an 8-year follow-up. A mother who developed IDDM during our study was negative for ICA and GAD antibodies at 6 and 3 years prior to clinical onset (at the age of 37), but positive for GAD antibodies at diagnosis, while ICA remained negative in all

samples. Conversion to antibody positivity later in life has also been described by other groups (50–53). Thus β-cell autoantibodies may appear early in life but conversion to antibody positivity later in life is possible, concomitant with both models of prediabetes. Antibody testing at one single moment during childhood will therefore be insufficient.

The increased frequencies and titres of IAA reported in young children raise the issue of whether there is a certain sequence in the appearance of β-cell autoantibodies. It has been observed that IAA may be transient in some young children, which might affect screening specificity in this group (7, 34). Bingley et al. described that several prediabetic siblings and parents went through an ICA-positive state before developing IAA (19), while Aanstoot et al. described GLIMA 38 and GAD antibodies as the earliest markers of autoimmunity (18). In our family population ICA and GAD antibodies were the first antibodies to appear in first-degree relatives. In the DAISY study most children went through a single antibody-positive state before development of multiple antibodies; these single antibodies were either GAD antibodies or IAA in all cases. Data on the 37/40 tryptic fragments of the 64K antigen (IA-2) suggest that IA-2 antibodies appeared closer to IDDM development (54, 55). At present, only data on the follow-up of first-degree relatives, but not in the general population, are available. We would also expect that in the general population β-cell autoantibodies do not appear simultaneously but in a certain, or possibly random, sequence.

In family-related studies, the number and titres of autoantibodies and the age of the individuals are the best predictors of imminent diabetes. We cannot yet use the pattern or titres of autoantibodies as surrogate endpoints of disease progression in individuals from the general population. In our own study of 1403 school children who were followed for 7 years (expected number of IDDM cases 1.4), two developed diabetes, 2 and 4 years after sampling. Both were among the three highest titres for GAD 65 antibodies and ICA, suggesting that, at least 2 and 4 years before onset, titres are also important predictors of imminent diabetes in the general population. However, sufficient data are lacking to establish that autoantibodies can be used as surrogate endpoints for diabetes development in the general population.

Screening Frequency

The question of when to screen remains to be answered satisfactorily and at present we suggest that repeated sampling is necessary. This approach would also provide information on the issue concerning the frequency of screening. In a study of 33 patients that were closely monitored during the first years after onset of IDDM we observed substantial fluctuations in GAD-antibody titres, while ICA were stable or absent. Seven patients seroconverted from GAD antibody negativity to positivity or vice versa during the first year of the disease (56). Although no correlation with clinical parameters was found, these observations raise the question of whether autoantibody fluctuations are correlated to immune activation and whether fluctuations occur before disease onset. Such fluctuations would support serial serological testing. Because of lack of large cohorts of prediabetic individuals the course of autoantibodies during the preclinical phase is not well-documented. In first-degree relatives ICA appear to remain stable when titres are high (≥20 JDFU) (57, 58). Others report that antibody levels are stable in relatives who are positive for multiple autoantibodies (7, 59, 60), which is associated with high ICA titres. IAA tend to disappear more often in initially positive individuals than other autoantibodies (57). Seroconversions occur mainly when antibody levels are low and in individuals who are positive for one antibody only (7, 51). In the general population transient positivity for ICA has also been observed, although high titres seem to be correlated to persistent positivity (51, 57). Thus, fluctuations in β-cell autoantibodies are observed in relatives as well as in the general population, but it is not yet established whether this is associated with a lower risk for clinical diabetes. Roll et al. described two children who were positive for multiple antibodies, including GAD antibodies, and became negative for GAD antibodies in the subsequent serum sample. One of these children developed IDDM after seroconversion (7). Other data on seroconversion in a prediabetic individual come from Bingley et al., who reported a prediabetic male who was GAD-antibody positive 8 years before disease onset but was found to be negative in subsequent samples taken before disease onset (19). In a cohort of unrelated Swedish children, stable antibody titres correlated with future IDDM, while children who converted from ICA positivity to negativity did not develop diabetes (6). Common viral infections may contribute to the formation of transient β-cell autoantibodies, without any correlation to future diabetes (61, 62). It is, however, conceivable that in some individuals β-cell destruction is indeed initiated, but that these individuals are able to stop the destructive process. The seroconversion to negative titres in prediabetic individuals who did develop IDDM may be caused by disruption of the T-helper 1 (TH1) and TH2 balance. Close to onset a more prominent TH1 response may correlate to a down-regulation of TH2 cells and reduced autoantibody production.

Of the 1403 schoolchildren we studied for GAD antibodies, five tested positive. During a 7-year follow-up two developed IDDM. Both were among the three with the highest GAD-antibody titres. Thus, a one-time high autoantibody titre did correlate with future IDDM. Follow-up studies will show whether the other three strongly positive individuals will progress to clinical diabetes. Finding sera that are repeatedly positive for β-cell antibodies may improve the specificity of autoantibody screening, but some loss in sensitivity must be taken into account. Extensive follow-up and more detailed knowledge on the natural course of autoimmunity is required before definite recommendations on the frequency of screening can be made. In conclusion, seroconversions are observed in prediabetic individuals. One positive autoantibody test may reflect β-cell

destruction, but is not necessarily corrected to imminent IDDM, further strengthening the need for repeated testing in population-based prediction studies.

Feasibility of Screening for Intervention in the General Population

Ongoing trials on prevention urge us to develop adequate prediction programmes for the general population. This requires a cost-effective screening system that is easy to implement in the general health care system. Normal 'rules', such as a voluntary basis and other requirements of the Helsinki Human Rights Declaration, are mandatory for any screening programme and will not be discussed. Repeated sampling in unaffected families may be difficult to achieve, in particular when increasing knowledge on the natural course of autoimmunity requires very young children to be screened by serial venipunctures. Compliance is likely to be best when such screening initiatives are integrated into the regular health care system (baby care, vaccination programmes etc.) and are simple (finger pricks rather than venipunctures). While ICA show acceptable specificity and sensitivity in first-degree family members, they are less applicable in the general population and tests for antibodies to molecularly defined targets such as GAD-65 and IA-2 require small sample sizes.

Figure 1 illustrates a proposed screening strategy. We limit this protocol to antibody screening. Genetic testing may precede this scheme. The model proposed in Figure 1 aims at both high sensitivity and specificity by repeated testing. The age of first antibody screening is dependent on the intervention strategy offered, but screening should not be performed before the age of 9 months to circumvent false-positive tests owing to maternal antibodies. A first antibody screening somewhere between the ages of 1 and 2 years is required because a substantial proportion of antibodies appear early in life, while diabetes incidence before the age of two years is low. This could be incorporated into regular visits to child welfare clinics. Multiple antibodies or high levels for single antibodies are likely to be persistent and confer high risk for IDDM. Therefore, once interventions have been shown to be effective and safe in young individuals, the individuals at high risk should be offered intervention. To improve sensitivity, a second antibody test within 1–4 years of the first should be offered to all other individuals. The peak incidence of diabetes is during puberty, therefore a third test performed shortly before puberty could select those who are persistently positive for one antibody only (in three successive tests) and those converting to multiple antibody positivity or high antibody titres. Because in

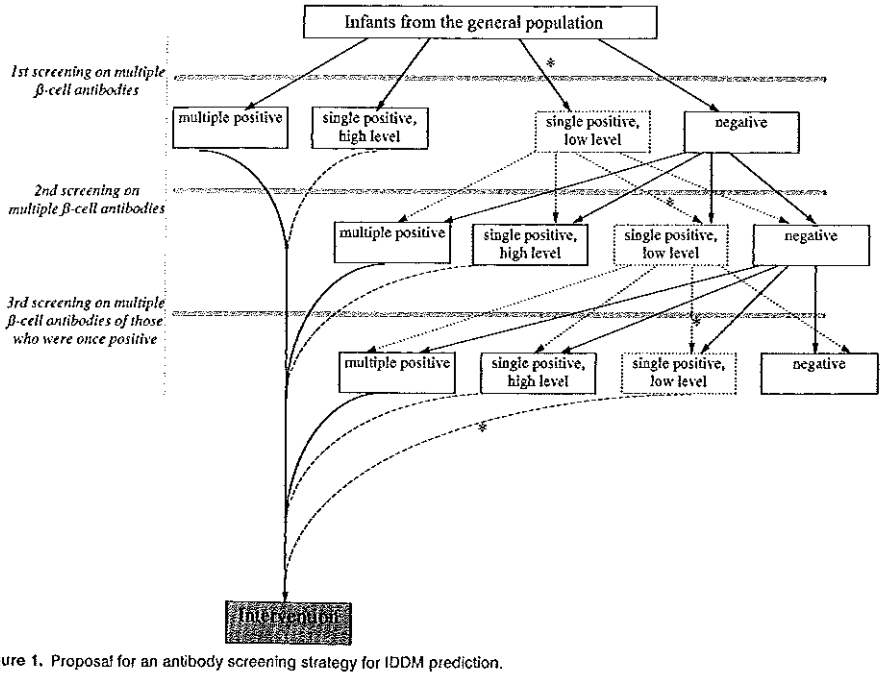

Figure 1. Proposal for an antibody screening strategy for IDDM prediction.

most countries regular health visits in official programmes (school etc.) stop after puberty, it is difficult to orchestrate screening in older individuals.

Thresholds for positivity and negativity and low and high antibody levels are the main determinants of the predictive value obtained by the proposed model. For GAD antibodies as well as IA-2 antibodies each laboratory uses its own standard sera, so applied thresholds for positivity in different laboratories are hardly comparable. To overcome these problems a similar system to that of JDF units for ICA standardization should be developed and implemented in the quality control system of the Immunology of Diabetes Society. In several laboratories the threshold for antibody positivity is set at the mean+standard deviations of a limited control group, while the statistical characteristics (non-bimodal distribution) of these groups do not always allow for such an analysis. When studying large groups of patients and healthy controls receiver-operating characteristic (ROC) analysis is an established technique for the optimization of assay thresholds (63). In most analyses sera of newly diagnosed patients are used to determine sensitivity of an assay. Epitope recognition of antibodies early in the prediabetic phase may, however, differ from recognition after clinical manifestation. Prediabetic individuals may therefore show lower responses in antibody assays and using sera of newly diagnosed patients to establish a threshold for IDDM may result in loss of assay sensitivity. Accordingly, prediabetic sera should be included in ROC analyses.

In our model (Fig. 1) the number of different autoantibodies rather than a particular autoantibody specificity determines the risk of IDDM (45). The type of autoantibodies (GAD, IA-2 or IAA) tested in the proposed model is irrelevant as long as testing of a large population is technically applicable. However, the transient nature and increased frequency of IAA at young age suggest that these antibodies are not suitable for screening early in life. Thus we would suggest that GAD 65 and IA-2 are used as antigens in the assays.

To reduce costs of population screening the cohort that should be tested for autoantibodies can be reduced by testing cord blood of all newborn infants for HLA susceptibility. Inclusion of all individuals positive for one or more susceptibility alleles will yield a maximum sensitivity (90%), selecting 10–50% (depending on tests and differences in frequency of susceptibility genes in different populations) of all infants for sequential antibody screening. These 10–50% include more than 90% of future cases with IDDM. Simell et al. have described that a substantial reduction of costs can be achieved by such a genetic preselection. However, in their genetic screening only high-risk individuals were selected, leading to a decrease in the sensitivity to only 60–70% (64).

The psychosocial and economic effects of the implementation of a screening and intervention programme should be carefully considered. At present, limited studies are available on the cost-effectiveness of diabetes prevention. Data from the USA suggest that one in every seven dollars spent in health care is related to the direct or indirect costs of diabetes. While this is calculated for both types of diabetes, the impact of IDDM alone is substantial and it is probable that prediction will ultimately become cost-effective. Being at risk of a disease may have serious consequences for health insurance or employment and may be a reason to refrain from testing. We studied the effects of prediction in 32 families. Only half of the families wished to know the outcome of prediction tests, in particular because no definite intervention is available (65). Fear for problems with insurance and jobs was another obstacle identified. People in the general population, who have no close relative with the disease, might have other motives for screening than people who are aware of the burden of the disease. Therefore, further studies on the psychological impact of IDDM screening and intervention in relatives and in the general population are required.

Conclusions

Within approximately five years the results of the first large-scale diabetes intervention trials will be available. If intervention appears to be effective in first-degree relatives, screening and prevention in the general population will become inevitable. It is now time to obtain data and knowledge on prediction in the general population, which has become technically possible. Several problems remain to be solved. Among these is the need to standardize assays between different laboratories and the need to determine thresholds of the assays based on adequate large-scale studies and follow-up studies. Predictive values of currently applied assays are predominantly calculated from family-based populations and the validity of extrapolation to the general population is dubious.

The lack of knowledge on the natural course of β-cell autoimmunity hampers the development of a strategy regarding who, when and how frequently we should screen in the general population. Currently, screening at multiple timepoints for multiple autoantibodies has the highest predictive value in most studies. While different mechanisms of β-cell autoimmunity may exist, we would presently suggest the use of repeated testing and extensive follow-up. The disadvantages of such a strategy are the expenses and the possible high drop-out rate from the screening programme. Identification of new genetic markers might enable better preselection of those at risk and genetic screening could even replace the first autoantibody screening as proposed in this review.

References

1. **Bingley PJ, Bonifaco E, Gale EA.** Can we really predict IDDM? *Diabetes* 1993; 42: 213–20.
2. **Bingley PJ.** Interactions of age, islet cell antibodies, insulin autoantibodies, and first-phase insulin response in

predicting risk of progression to IDDM in ICA+relatives: the ICARUS data set. Islet Cell Antibody Register Users Study. *Diabetes* 1996; 45: 1720–8.

3. **Schatz D, Krischer J, Horne G, et al.** Islet cell antibodies predict insulin-dependent diabetes in United States school age children as powerfully as in unaffected relatives. *J Clin Invest* 1994; 93: 2403–7.
4. **Bingley PJ, Bonifacio E, Shattock M, et al.** Can islet cell antibodies predict IDDM in the general population? *Diabetes Care* 1993; 16: 45–50.
5. **Elliott RB, Pilcher CC.** A universal model of antibody prediction (MAP) of type I diabetes in children. *J Autoimmun* 1994; 7: 881–8.
6. **Hagopian WA, Sanjeevi CB, Kockum I, et al.** Glutamate decarboxylase-, insulin-, and islet cell-antibodies and HLA typing to detect diabetes in a general population-based study of Swedish children. *J Clin Invest* 1995; 95: 1505–11.
7. **Roll U, Christie MR, Fuchtenbusch M, Payton MA, Hawkes CJ, Ziegler AG.** Perinatal autoimmunity in offspring of diabetic parents. The German Multicenter BABY-DIAB study: detection of humoral immune responses to islet antigens in early childhood. *Diabetes* 1996; 45: 967–73.
8. **Ziegler AG, Hillebrand B, Rabl W, et al.** On the appearance of islet associated autoimmunity in offspring of diabetic mothers: a prospective study from birth. *Diabetologia* 1993; 36: 402–8.
9. **Knip M, Vähäsalo P, Karjalainen J, Lounamaa R, Åkerblom HK.** Natural history of preclinical IDDM in high risk siblings. Childhood Diabetes in Finland Study Group. *Diabetologia* 1994; 37: 388–93.
10. **Balsch JM, Weeks T, Giles R, Hoover M, Stastny P, Capra JD.** Analysis of HLA-DQ genotypes and susceptibility in insulin-dependent diabetes mellitus. *N Engl J Med* 1990; 322: 1836–41.
11. **Veijola R, Knip M, Reijonen H, Vähäsalo P, Puukka R, Ilonen J.** Effect of genetic risk load defined by HLA-DQB1 polymorphism on clinical characteristics of IDDM in children. *Eur J Clin Invest* 1995; 25: 106–12.
12. **Pociot F, Ronningen KS, Bergholdt R, et al.** Genetic susceptibility markers in Danish patients with type 1 (insulin-dependent) diabetes – evidence for polygenicity in man. Danish Study Group of Diabetes in Childhood. *Autoimmunity* 1994; 19: 169–78.
13. **Todd JA, Farrall M.** Panning for gold: genome-wide scanning for linkage in type 1 diabetes. *Hum Mol Genet* 1996; 5: 1443–8.
14. **Merriman TR, Todd JA.** Genetics of insulin-dependent diabetes; non-major histocompatibility genes. *Horm Metab Res* 1996; 28: 289–93.
15. **Homo-Delarche F, Boitard C.** Autoimmune diabetes: the role of the islets of Langerhans. *Immunol Today* 1996; 17: 456–60.
16. **Vyse TJ, Todd JA.** Genetic analysis of autoimmune disease. *Cell* 1996; 85: 311–8.
17. **Sigurdsson E, Baekkeskov S.** The 64-kDa beta cell membrane autoantigen and other target molecules of humoral autoimmunity in insulin-dependent diabetes mellitus. *Curr Top Microbiol Immunol* 1990; 164: 143–68.
18. **Aanstoot HJ, Kang SM, Kim J, et al.** Identification and characterization of glima 38, a glycosylated islet cell membrane antigen, which together with GAD65 and IA2 marks the early phases of autoimmune response in type 1 diabetes. *J Clin Invest* 1996; 97: 2772–83.
19. **Bingley PJ, Christie MR, Bonifacio E, et al.** Combined analysis of autoantibodies improves prediction of IDDM in islet cell antibody-positive relatives. *Diabetes* 1994; 43: 1304–10.
20. **Bonifacio E, Genovese S, Braghi S, et al.** Islet autoantibody markers in IDDM: risk assessment strategies yielding high sensitivity. *Diabetologia* 1995; 38: 816–22.
21. **Aanstoot HJ, Sigurdsson E, Jaffe M, et al.** Value of antibodies to GAD65 combined with islet cell cytoplasmic antibodies for predicting IDDM in a childhood population. *Diabetologia* 1994; 37: 917–24.
22. **Boehm BO, Manfras B, Seissler J, et al.** Epidemiology and immunogenetic background of islet cell antibody-positive nondiabetic schoolchildren. Ulm–Frankfurt population study. *Diabetes* 1991; 40: 1435–9.
23. **Landin-Olsson M, Palmer JP, Lernmark A, et al.** Predictive value of islet cell and insulin autoantibodies for type 1 (insulin-dependent) diabetes mellitus in a population-based study of newly diagnosed diabetic and matched control children. *Diabetologia* 1992; 35: 1068–73.
24. **Samuelsson U, Ludvigsson J, Sundkvist G.** Islet cell antibodies (ICA), insulin autoantibodies (IAA), islet cell surface antibodies (ICSA) and C-peptide in 1031 school children in a population with a high background incidence of IDDM. *Diabetes Res Clin Pract* 1994; 26: 155–62.
25. **Levy-Marchal C, Tichet J, Fajardy I, Gu XF, Dubois F, Czernichow P.** Islet cell antibodies in normal French schoolchildren. *Diabetologia* 1992; 35: 577–82.
26. **Bruining GJ, Molenaar JL, Grobbee DE, et al.** Ten-year follow-up study of islet-cell antibodies and childhood diabetes mellitus. *Lancet* 1989; 1: 1100–3.
27. **Boitard C, Bonifacio E, Bottazzo GF, Gleichmann H, Molenaar J.** Immunology and Diabetes Workshop: report on the Third International (Stage 3) Workshop on the Standardisation of Cytoplasmic Islet Cell Antibodies. New York, October 1987. *Diabetologia* 1988; 31: 451–2.
28. **Lernmark A, Molenaar JL, van Beers WA, et al.** The Fourth International Serum Exchange Workshop to standardize cytoplasmic islet cell antibodies. The Immunology and Diabetes Workshops and Participating Laboratories. *Diabetologia* 1991; 34: 534–5.
29. **Greenbaum CJ, Palmer JP, Nagataki S, et al.** Improved specificity of ICA assays in the Fourth International Immunology of Diabetes Serum Exchange Workshop. *Diabetes* 1992; 41: 1570–4.
30. **Gorus FK, Goubert P, Semakula C, et al.** IA-2-autoantibodies complement GAD65-autoantibodies in new-onset IDDM patients and help predict impending diabetes in their siblings. The Belgian Diabetes Registry *Diabetologia* 1997; 40: 95–9.
31. **Christie MR, Genovese S, Cassidy D, et al.** Antibodies to islet 37 k antigen, but not to glutamate decarboxylase, discriminate rapid progression to IDDM in endocrine autoimmunity. *Diabetes* 1994; 43: 1254–9.
32. **Wiesladenburger U, Hartmann R, Hartmann U, Berling K, Bohm BO, Richter W.** Combined analysis and single-step detection of GAD65 and IA2 autoantibodies in IDDM can replace the histochemical islet cell antibody test. *Diabetes* 1997; 46: 565–71.
33. **Palmer JP, Asplin CM, Clemons P, et al.** Insulin antibodies in insulin-dependent diabetics before insulin treatment. *Science* 1983; 222: 1337–9.
34. **Ziegler AG, Hillebrand B, Rabl W, et al.** On the appearance of islet associated autoimmunity in offspring of diabetic mothers: a prospective study from birth. *Diabetologia* 1993; 36: 402–8.
35. **Vähäsalo P, Knip M, Karjalainen J, Tuomilehto-Wolf E, Lounamaa R, Åkerblom HK.** Islet cell-specific autoantibodies in children with insulin-dependent diabetes mellitus and their siblings at clinical manifestation of the disease. Childhood Diabetes in Finland Study Group. *Eur J Endocrinology* 1996; 135: 689–95.
36. **Karjalainen J, Knip M, Mustonen A, Ilonen J, Åkerblom HK.** Relation between insulin antibody and complement-fixing islet cell antibody at clinical diagnosis of IDDM. *Diabetes* 1986; 35: 620–2.
37. **Vardi P, Ziegler AG, Mathews JH, et al.** Concentration of insulin autoantibodies at onset of type I diabetes: inverse

log-linear correlation with age. *Diabetes Care* 1988; 11: 736–9.

38. **Baekkeskov S, Aanstoot HJ, Christgau S, et al.** Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. *Nature* 1990; 347: 151–6.
39. **Lan MS, Wasserfall C, Maclaren NK, Notkins AL.** IA-2, a transmembrane protein of the protein tyrosine phosphatase family, is a major autoantigen in insulin-dependent diabetes mellitus. *Proc Natl Acad Sci USA* 1996; 93: 6367–70.
40. **Hawkes CJ, Wasmeier C, Christie MR, Hutton JC.** Identification of the 37-kDa antigen in IDDM as tyrosine phosphatase-like protein (phogrin) related to IA-2. *Diabetes* 1996; 1187–92.
41. **Gianani R, Rabin DU, Verge CF, et al.** ICA512 autoantibody radioassay. *Diabetes* 1995; 44: 1340–4.
42. **Lampasona V, Bonifacio E, Bingley P, Gale E.** Development of IA-2 autoantibody epitope specifities in sequential samples from relatives. *Autoimmunity* 1996; 24 (S1): A028.
43. **Genovese S, Bingley PJ, Bonifacio E, et al.** Combined analysis of IDDM-related autoantibodies in healthy schoolchildren (letter). *Lancet* 1994; 344: 756.
44. **Bonifacio E, Lampasona V, Genovese S, Ferrari M, Bosi E.** Identification of protein tyrosine phosphatase-like IA2 (islet cell antigen 512) as the insulin-dependent diabetes-related 37/40K autoantigen and a target of islet-cell antibodies. *J Immunol* 1995; 155: 5419–26.
45. **Verge CF, Gianani R, Kawasaki E, et al.** Number of autoantibodies (against insulin, GAD or ICA512/IA2) rather than particular autoantibody specificities determines risk of type I diabetes. *J Autoimmun* 1996; 9: 379–83.
46. **Norris JM, Beaty B, Klingensmith G, et al.** Lack of association between early exposure to cow's milk protein and beta-cell autoimmunity. Diabetes Autoimmunity Study in the Young (DAISY). *JAMA* 1996; 276: 609–14.
47. **Rewers M, Bugawan TL, Norris JM, et al.** Newborn screening for HLA markers associated with IDDM: diabetes autoimmunity study in the young (DAISY). *Diabetologia* 1996; 39: 807–12.
48. **Roll U, Scheeser J, Standl E, Ziegler AG.** Alterations of lymphocyte subsets in children of diabetic mothers. *Diabetologia* 1994; 37: 1132–41.
49. **Martikainen A, Saukkonen T, Kulmala PK, et al.** Disease-associated antibodies in offspring of mothers with IDDM. *Diabetes* 1996; 45: 1706–10.
50. **Bonifacio E, Bingley PJ, Shattock M, et al.** Quantification of islet-cell antibodies and prediction of insulin-dependent diabetes. *Lancet* 1990; 335: 147–9.
51. **Roll U, Christie MR, Standl E, Ziegler AG.** Associations of anti-GAD antibodies with islet cell antibodies and insulin autoantibodies in first-degree relatives of type I diabetic patients. *Diabetes* 1994; 43: 154–60.
52. **Riley WJ, Maclaren NK, Krischer J, et al.** A prospective study of the development of diabetes in relatives of patients with insulin-dependent diabetes. *N Engl J Med* 1990; 323: 1167–72.
53. **Yu L, Rewers M, Gianani R, et al.** Antiislet autoantibodies usually develop sequentially rather than simultaneously. *J Clin Endocrinol Metab* 1996; 81: 4264–7.
54. **Christie MR, Vohra G, Champagne P, Daneman D, Delovitch TL.** Distinct antibody specificities to a 64-kD islet cell antigen in type 1 diabetes as revealed by trypsin treatment. *J Exp Med* 1990; 172: 789–94.
55. **Payton MA, Hawkes CJ, Christie MR.** Relationship of the 37,000- and 40,000-M(r) tryptic fragments of islet antigens in insulin-dependent diabetes to the protein tyrosine phosphatase-like molecule IA-2 (ICA512). *J Clin Invest* 1995; 96: 1506–11.
56. **Batstra MR, Pina M, Quan J, et al.** Fluctuations in GAD (65) antibodies after clinical diagnosis of IDDM in young children. *Diabetes Care* 1997; 20: 642–4.
57. **Levy-Marchal C, Dubois F, Noel M, Tichet J, Czernichow P.** Immunogenetic determinants and prediction of IDDM in French schoolchildren. *Diabetes* 1995; 44: 1029–32.
58. **Hiltunen M, Hyöty H, Karjalainen J, et al.** Serological evaluation of the role of cytomegalovirus in the pathogenesis of IDDM: a prospective study. The Childhood Diabetes in Finland Study Group. *Diabetologia* 1995; 38: 705–10.
59. **Schmidli RS, DeAizpurua HJ, Harrison LC, Colman PG.** Antibodies to glutamic acid decarboxylase in at-risk and clinical insulin-dependent diabetic subjects: relationship to age, sex and islet cell antibody status, and temporal profile. *J Autoimmun* 1994; 7: 55–66.
60. **Simone E, Eisenbarth GS.** Chronic autoimmunity of type 1 diabetes. *Horm Metab Res* 1996; 28: 332–36.
61. **Bodansky HJ, Grant PJ, Dean BM, et al.** Islet-cell antibodies and insulin autoantibodies in association with common viral infections. *Lancet* 1986; 2: 1351–3.
62. **Hyöty H, Hiltunen M, Knip, et al.** A prospective study of the role of coxsackie B and other enterovirus infections in the pathogenesis of IDDM. Childhood Diabetes in Finland (DiMe) Study Group. *Diabetes* 1995; 44: 652–6.
63. **Zweig MH, Campbell G.** Receiver-operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine. *Clin Chem* 1993; 39: 561–77.
64. **Simell T, Hahl J, Muona P, Simell O.** Direct costs of genetic prediction of IDDM and subsequent ICA follow-up of the at-risk children vs. direct costs of repeated unselected ICA screenings. *Autoimmunity* 1996; 24 (S1): A190.
65. **Wagner A, Tibben A, Bruining GJ, et al.** Preliminary experience with predictive testing for insulin-dependent diabetes mellitus (letter). *Lancet* 1995; 346: 380–1.

Chapter 3.2

Can we predict diabetes in schoolchildren? Threshold definition in GAD- and IA2 antibody assays

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Submitted

3.2.1. Abstract

3.2.1.1. Aims

Studies on prediction of type-1 diabetes mellitus generally aim at developing a strategy with both high sensitivity and specificity. However, the requirements on specificity and sensitivity of predictive screening should be defined according to the purpose of prediction. The current study evaluates the influence of threshold definition in radiobinding assays for GAD- and IA2-antibodies on screening efficiency in the general population.

3.2.1.2. Methods

1403 unselected schoolchildren, aged 10-12, and 244 children with newly diagnosed diabetes, aged 0-17, were tested for GAD- and IA2-antibodies by radiobinding assay. The antibody frequency applying different thresholds was established. Development of diabetes was recorded during 7 years follow-up.

3.2.1.3. Results

At the 99.5th centile threshold five individuals were positive for GAD-antibodies and one was positive for IA2-antibodies. Two children developed diabetes during follow-up, one was positive for GAD-antibodies only, the second was positive for both GAD- and IA2-antibodies. At Receiver Operated Curve (ROC) derived thresholds 102 (7%) and 51 (5%) individuals were positive for GAD- and IA2-antibodies respectively.

3.2.1.4. Conclusions

Application of different thresholds in radiobinding assays significantly influences the outcome of screening of the general population. In the current study ROC analysis results in low specificity of screening. Before embarking intervention trials in at risk individuals identified by antibody screening, agreement on threshold definition and assay standardization is essential.

3.2.2. Introduction

Type-1 diabetes mellitus is, except for asthma, the most prevalent chronic disease of childhood. The disease is accompanied by serious morbidity, due to long term micro- and macrovascular complications. The cost of intensive diabetes treatment, which is essential for the prevention or delay of these complications, is estimated at US$ 4000 per patient per year [1]. These facts urge for prevention of type-1 diabetes. This requires, in addition to effective preventive measures, efficient identification of individuals at increased risk to develop the disease.

Type-1 diabetes mellitus is preceded by a preclinical phase, which gives the opportunity for disease prediction and eventually intervention in the disease process. Prediction of type-1 diabetes mellitus by the detection of circulating autoantibodies has been extensively studied in first-degree relatives of patients with the disease. In these relatives high titers of multiple autoantibodies confer a 5 years diabetes-risk of 50–80% [2-4]. Current trials on prevention of type-1 diabetes mellitus are based on such risk-estimates [5, 6]. While only first degree relatives are included in these trials, over ninety percent of new cases with type-1 diabetes do not have a relative with the disease. The predictive value of antibody screening is estimated to be tenfold lower in the general population than in first-degree relatives [7, 8]. Substantial reduction of the incidence of type-1 diabetes will only be achieved when preventive measures are applicable to the general population. Therefore, predictive screening for type-1 diabetes in the general population needs to be evaluated.

Studies on diabetes prediction aim at the development of the best prediction strategy, being both highly sensitive and specific. Since any such strategy is a trade-off between these two important test characteristics, the aims of prediction should be defined before establishing a screening strategy. A highly sensitive and therefore less specific strategy is for example required for a follow-up study into the natural course of auto-immunity during pre-diabetes, while for highly

effective intervention with large potential side effects specificity is important. Thus, there is no universal best diabetes prediction strategy; it should be adapted to its aims.

Screening strategies for the general population that have been described until now are mostly based on initial testing for islet cell antibodies (ICAs), followed by screening for other antibodies [6-11]. Several studies in newly diagnosed patients demonstrate that screening for a combination of antibodies against glutamic acid decarboxylase (GAD) and IA2-antibodies can replace ICA screening [9, 11-15]. In the Immunology of Diabetes Society (IDS) workshops screening for these antibodies by radio-binding assays has shown to be highly reproducible [16]. In addition, the method can be (semi-)automated, applied using different autoantigens simultaneously and requires only small volumes of blood (<10µl) [13, 14, 17].

Despite the organization of workshops, comparison of tests between different laboratories is still hampered by lack of standardization and application of different thresholds. This results in large differences in the reported prevalence of autoantibodies. Bingley and colleagues reported 3% prevalence of GAD- or IA2-antibodies in first line screening of a general population [18]. Such relatively high prevalence in comparison to the incidence of type-1 diabetes in this population (18.6 per 100.000 per year [19]) urges to improve screening specificity.

In the present study we evaluated the effect of threshold definition on the outcome of screening for GAD- and IA2-antibdodies in a population of 1403 schoolchildren using both statistical methods and a technical evaluation of the radiobinding assays. The population studied was unselected and seven years follow-up was available.

3.2.3. Patients and methods

Sera from 1403 unselected schoolchildren were collected in 1987, stored at -70°C and not thawed prior to analysis. The family history for type-1

diabetes and type-2 diabetes was recorded. In 1995 the development of diabetes was ascertained through the regional diabetes registry (coverage >96%) [20] and scrutinized by checking the general practitioners, pediatric and internal medicine records. Eighteen individuals (1.3%) moved during follow-up. The population has been described in detail elsewhere [21]. Informed consent was obtained from all participants.

GAD-antibodies were determined in serum by radiobinding assay using in vitro translated ^{35}S-methionine labeled human recombinant GAD_{65} as tracer [22]. The test scored 100% sensitivity and specificity in the 1995 IDS proficiency program. Two negative, one positive and a blank control were included in every third plate. All samples were analyzed in triplicate in a period of two days, using one batch of tracer.

One-thousand-eighty-five of 1403 serum samples were available for IA2-antibody analysis. IA2-antibodies were tested in a radiobinding assay using in vitro translated ^{35}S-methionine labeled intracellular domain of IA2 (AA 603-980; a gift from Dr. M. Christie, London, UK) as tracer [23]. In this test 132 of 235 newly diagnosed patients (56%) were positive for IA2-antibodies. A mix of sera of three newly diagnosed patients, who were positive for IA2-antibodies, and a 1:64 dilution of this mix were used as positive controls in each plate. The diluted serum precipitated slightly more than the 99.5th centile of normal sera, and was used as an internal control of assay performance. In addition, two negative control sera were included in each plate. All samples were analyzed in triplicate in a period of 1one day, using one batch of tracer.

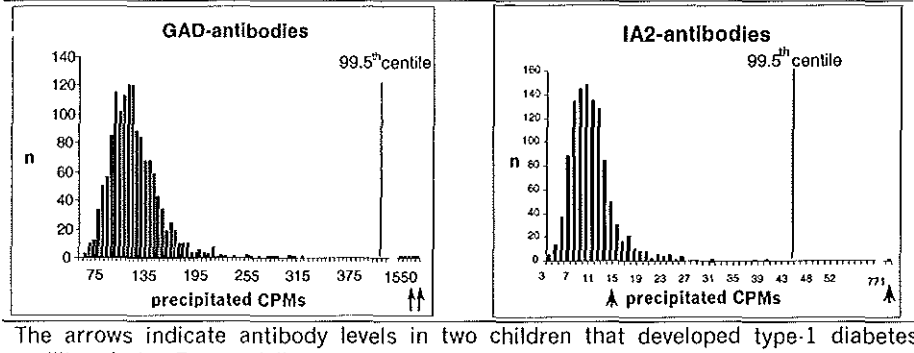
In both assays precipitated tracer was analyzed in a Microbeta plate reader (EG&G Wallac, Turku, Finland) and expressed as CPMs. To correct for variation between testplates an index was calculated, using the undiluted positive and the negative control sera as internal reference [22]. Receiver operated curve (ROC) analysis was used to establish assay thresholds. In the ROC analysis an additional set of sera, drawn from 244 (mean age 8.8, 114 males) and 235 (mean age 8.8, 110 males) newly

diagnosed type-1 diabetes patients (maximum disease duration: two weeks), was used to estimate assay sensitivity for GAD- and IA2-antibodies respectively.

For GAD-antibodies the results of all samples above the mean + 1 SD of precipitated CPMs and an equal number of negative samples were confirmed by competition analysis using unlabeled human recombinant GAD_{65} as well as by immunoprecipitation followed by SDS-PAGE and fluorography [24]. For IA2-antibodies, all samples above the 99.5th centile were confirmed using immunoprecipitation followed by SDS-PAGE and fluorography [24].

All samples that precipitated more than the mean + 1 SD of GAD-antibodies (n=30) or more than the 99.5th centile of IA2-antibodies and a sample of 25 random GAD- and IA2-antibody negative sera were tested for ICAs [25].

Figure 3.2.1. Distribution of GAD-antibodies (panel A) and IA2A (panel B) in a population of 1403 and 1085 unselected schoolchildren respectively.



The arrows indicate antibody levels in two children that developed type-1 diabetes mellitus during 7 years follow-up.

3.2.4. Results

3.2.4.1. Antibody tests – statistical evaluation

The distributions and assay characteristics of GAD- and IA2-antibodies are shown in figure 3.2.1. and table 3.2.1., respectively.

Table 3.2.1. Characteristics of radiobinding assay for GAD- and IA2-antibodies.

	GAD-antibodies		IA2-antibodies	
	CPMs	Index	CPMs	Index
Median	101	-0.018	9	0.0012
Mean positive control (CV)	1396 (24%)	1	927 (10%)	1
Mean diluted positive control	-	-	84	0.08
Mean negative control (CV)	116 (20%)	0	8 (22%)	0
99.5th centile	395	0.21	44	0.038
ROC threshold	175	0.04	15	0.007

CPMs indicate precipitated tracer. Index indicates the antibody index that is calculated to correct for interplate and interassay variation.

Using the 99.5th centile of precipitated CPMs as threshold, five individuals (0.4%) were positive for GAD-antibodies. Applying the 99.5th centile of the GAD-index as threshold resulted in four positive sera. These were the sera that scored highest in the CPM distribution.

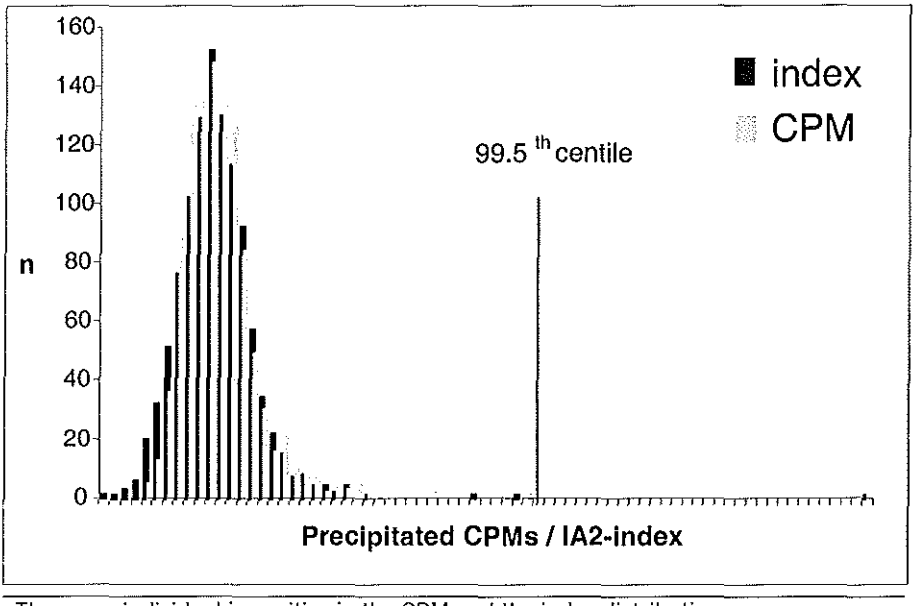
Using the optimal cut-off as determined by receiver operated curve (ROC) analysis resulted in assay sensitivity of 66% and specificity of 93%, thus identifying 102 of 1403 schoolchildren as positive.

From 1085 sera available for IA2-antibody testing (including all sera that were positive for GAD-antibodies and ICA) one serum precipitated more than the 99.5th centile of precipitated CPMs. The CPM distribution and the IA2-index distribution revealed the same individual as positive. As shown in figure 3.2.2., the distributions of IA2 CPMs and IA2-index were similar, indicating that a correction for interplate variation is not necessary when samples are tested in one test-run with one batch of tracer.

Using the threshold as established by the ROC-analysis resulted in 75% sensitivity and 95% specificity, identifying 51 children positive (table 3.2.2.). Applying the ROC derived thresholds in a combined analysis for GAD- and IA2-antibodies resulted in 10 positive individuals (1%, table 3.2.2.). Using the Spearman's correlation test a weak, but significant correlation between GAD- and IA2-antibody levels was observed, both when considering only the samples with a test result below the 99.5th centile of the GAD- and IA2-antibody test (R=0.088, p =0.005) and

considering all samples (R=0.097 p <0.001). Only three individuals were positive for ICAs, those were the ones with the highest GAD-antibodies levels (table 3.2.3.).

Figure 3.2.2. Comparison of the distribution of IA2-antibodies expressed as IA2-index and precipitated CPMs in 1085 schoolchildren



The same individual is positive in the CPM and the index-distribution.

3.2.4.2. Antibody tests – technical evaluation

The results of the immunoprecipitations evaluated by SDS-PAGE and fluorography are shown in table 3.2.3. All sera that precipitated more than the 99.5th centile in the GAD- and IA2-antibody assay were positive in the corresponding SDS-PAGE experiment and no additional positive samples were found. This confirms the results of the radiobinding assay (table 3.2.3.). Competition analysis using unlabeled recombinant human GAD_{65} inhibited precipitation in all GAD-antibody positive samples, but did not discriminate between samples just below the 99.5th centile and those that precipitated more than the 99.5th centile.

Table 3.2.2. Characteristics of combined and single predictive screening for GAD- and IA2-antibodies, applying different thresholds.

99.5th centile threshold

A

	DM+	DM-	
GADA+	2	3	*5*
GADA-	0	1398	*1398*
	2	*1401*	*1403*

Sens: 100%
Spec: 99.7%
PPV: 40%

C

	DM+	DM-	
IA2A+	1	0	1
IA2A-	1	1083	*1084*
	2	*1083*	*1085*

Sens: 50%
Spec: 100%
PPV: 100%

E

	DM+	DM-	
2Ab +	1	0	*1*
≤1Ab +	1	1083	*1084*
	2	*1083*	*1085*

Sens: 50%
Spec: 100%
PPV: 100%

ROC derived threshold

B

	DM+	DM-	
GADA+	2	100	*102*
GADA-	0	1301	*1301*
	2	*1401*	*1403*

Sens: 100%
Spec: 92.8%
PPV: 1.9%

D

	DM+	DM-	
IA2A+	1	50	*51*
IA2A-	1	1033	*1034*
	2	*1083*	*1085*

Sens: 50%
Spec: 95%
PPV: 2%

F

	DM+	DM-	
2Ab +	1	9	*10*
≤1Ab +	1	1074	*1075*
	2	*1083*	*1085*

Sens: 50%
Spec: 99%
PPV: 10%

Number of positive schoolchildren and predictive value applying the 99th centile (A,C,E) and ROC derived thresholds (B,D,F) for GAD-antibodies (GADA) (A,B) and IA2-antibodies (IA2A) (C,D) separately and a combination of both (E,F). Specificity (Spec) of screening is greatly impaired when applying the ROC derived thresholds, while sensitivity (sens) is not affected. PPV: postive predictive value. 2Ab+: positive for GAD- and IA2-antibodies. ≤1Ab: positive for GAD- or IA2-antibodies or negative for all antibodies tested.

3.2.4.3. Occurrence of diabetes

Data from the regional diabetes registry and medical records revealed that two children out of the cohort (0,14%) developed diabetes during seven years follow-up (table 3.2.3.). The number of cases expected in this cohort during this period was 1.4. Both cases were female and positive for GAD-antibodies. The individual with the highest GAD-antibody level (2115 CPMs, mean + 24 SD) developed diabetes 3 years after sampling. This individual was positive for ICAs, but negative for IA2-antibodies. Her family

history for diabetes was negative. The second case who had the third highest GAD-antibody level (1474 CPMs, mean + 16 SD) developed diabetes 4.5 years after sampling. She was positive for IA2-antibodies and ICAs. The remaining three GAD-antibody positive individuals were still healthy in 1997 and had no family history for type-1 diabetes.

Table 3.2.3. Characteristics of GAD-antibody positive schoolchildren.

	GAD-antibodies		IA2-antibodies		ICA	T-1 DM
ID	RBA	G	RBA	G		
1	2115	+	11.1	·	+	+
2	1546	+	9.8	·	+	·
3	1474	+	771.0	+	+	+
4	633	+	25.8	·	·	·
5	419	+	16.7	·	·	·

GAD- and IA2-antibodies were tested in radiobinding assay (RBA) and conventional immunoprecipitations evaluated by SDS-PAGE and fluorography (G). Development of type-1 diabetes (T-1 DM) was recorded during 7 years follow-up.

3.2.5. Discussion

The results of the first diabetes prevention trials are expected within five years [5, 6]. In these trials first degree relatives of patients with type-1 diabetes are included, but substantial reduction of diabetes incidence can only be achieved if preventive measures are applicable to the general population. Therefore, the development of reliable prediction tools for the general population is a prerequisite.

This study aims at autoantibody screening only, but the application of genetic markers (HLA-DQ susceptibility and protective haplotypes) as a first sieve to select approximately 10% of the general population that is susceptible to type-1 diabetes may decrease screening costs and improve screening specificity [26, 27]. The specificity of genetic screening is low and therefore additional screening for antibody markers is required to improve screening specificity. Since the development of radiobinding assays for GAD- and IA2-antibodies this has become technically feasible [5, 6].

The reported prevalence of antibodies in the general populations is highly variable between different countries. The variation might not just reflect

differences between populations, but may also be due to application of different test and thresholds. In addition, the frequency of antibodies generally exceeds the diabetes prevalence, indicating that antibody screening is not 100% specific [8, 18]. Adjustment of assay-thresholds may improve specificity of screening and should be standardized internationally [17].

The present study evaluates the effect of threshold definition in the radiobinding assays for GAD- and IA2-antibodies on predictive screening in 1403 schoolchildren. During seven years follow-up two children developed type-1 diabetes mellitus. Although this number is too low to calculate the predictive power of different antibodies and antibody-combinations, the study enables us to estimate the efficacy of population screening.

3.2.5.1. Statistical evaluation of diabetes prediction and assay thresholds

At the 99.5th centile, a threshold that could be confirmed technically in conventional immunoprecipitations, screening for GAD-antibodies was 100% sensitive and 99.7% specific. Screening for IA2-antibodies was 100% specific at the cost of low sensitivity (50%) (table 3.2.2.). To improve sensitivity Bingley and colleagues proposed to lower the threshold in the IA2-antibody assay to the 95th centile (14 CPMs in our population) [18]. In the current population this does not improve prediction. At the lower threshold the second prediabetic individual is still not positive, while the specificity is seriously impaired, yielding 52 false positive individuals.

The optimum thresholds established by ROC analysis yielded 66% and 75% sensitivity and 93% and 95% specificity in the GAD- and IA2-antibody tests, respectively. This means that, if diabetes intervention trials would be based on predictive screening using these ROC derived thresholds, 100 and 50 individuals would be unjustly treated, respectively (table 3.2.2.). As shown in table 3.2.2. combined analysis applying ROC derived thresholds results in better prediction (50% sensitivity, 99% specificity), but still the

predictive value in this population is only 10%. The impact of these figures on population screening is demonstrated by the Bayes theorem. In a family based population, with a diabetes prevalence of 15/1000, the probability to develop diabetes for an individual who is positive for GAD- and IA2-antibodies (at the ROC derived threshold) is 43%. Since the diabetes prevalence in the general population is lower (0.77/1000), a similar individual without a family history for diabetes would have a tenfold lower risk to develop the disease (4%) (table 3.2.4.). One could question if treatment of individuals based on these risk estimates is ethically justified.

Table 3.2.4. Posterior chance according to Bayes in family- and population-based predictive screening for type-1 diabetes.

Threshold	Relatives	General population
GADA > 99.5th centile	83%	20%
IA2A > 99.5th centile	100%	100%
GADA > ROC derived threshold	17%	1%
IA2A > ROC derived threshold	14%	0.7%
GADA and IA2A > 99.5th centile	100%	100%
GAD and IA2A > ROC derived threshold	40%	4%

In this population screening for high levels (>99.5th centile) of IA2-antibodies results in 100% posterior chance in both the general population and family-based populations. However, this is accompanied by low sensitivity (50%) of screening. The posterior chance using other antibodies or thresholds is significantly smaller, but sensitvity may be higher (compare to table 3.2.2.).
GADA: GAD-antibodies IA2A: IA2-antibodies

Receiver operated curve (ROC) analysis aims to maximize both sensitivity and specificity and finds a compromise between these two screening parameters, regardless of the aims of screening. Similar to what is done in other studies [13, 18, 28], we performed ROC analysis, using sera of newly diagnosed patients to establish assay sensitivity. Such an analysis is based on the assumption that antibodies before onset are identical to antibodies at diagnosis of type-1 diabetes. However, due to the longer existence of autoimmunity, the immune recognition in newly diagnosed patients may significantly differ from the reaction in recently initiated autoimmunity during prediabetes [29, 30]. This may seriously influence the results of the ROC analysis. Therefore, studies on prediabetic individuals

are essential for proper design of diabetes intervention trials. Studies on the course of antibodies during prediabetes and differences between samples taken before and after onset of diabetes will both reveal insight into pathogenesis of type-1 diabetes and improve diabetes prediction.

3.2.5.2. Technical evaluation of assay thresholds

As an alternative method to establish assay thresholds we compared the results of the radiobinding assay for both antibodies to the conventional assays using SDS-PAGE and fluorography. This conventional method has the advantage of extremely low background levels, and identification of the precipitated antigen is unambiguous because only precipitated radioactivity of relevant molecular weight is measured [31]. The conventional immunoprecipitations and the 99.5th centile of the CPM distribution in the radiobinding assays identified the same individuals as positive for both the IA2- and the GAD-antibody assays. Therefore, these thresholds were used for further analysis. In future studies confirmation of positive and dubious sera in the radiobinding assays by conventional immunoprecipitations will improve screening specificity.

3.2.5.3. Conclusion

Definition of thresholds in radiobinding assays for autoantibodies is difficult and should be seen in the context of the specific aims of diabetes prediction. Our data demonstrate that ROC analysis using newly diagnosed patients to establish assay sensitivity results in low assay specificity. Applying the 99.5th centile threshold in the radiobinding assays results in higher specificity and this threshold is confirmed by conventional immunoprecipitation and SDS-PAGE techniques. When high screening specificity is required, we propose to confirm the results of positive or borderline sera obtained in radiobinding assays by conventional techniques.

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References

1. The Diabetes Control and Complications Trial Research Group. Resource utilization and costs of care in the diabetes control and complications trial. *Diabetes Care* 1995;18(11):1468-78.
2. Seidel D, Ziegler AG. Prediction of type 1 diabetes. *Horm Res* 1996;45(Suppl 1):36-9.
3. Bingley PJ, Christie MR, Bonifacio E, Bonfanti R, Shattock M, Fonte MT, et al. Combined analysis of autoantibodies improves prediction of IDDM in islet cell antibody-positive relatives. *Diabetes* 1994;43(11):1304-10.
4. Verge CF, Gianani R, Kawasaki E, Yu L, Pietropaolo M, Jackson RA, et al. Prediction of type I diabetes in first-degree relatives using a combination of insulin, GAD, and ICA512bdc/IA-2 autoantibodies. *Diabetes* 1996;45(7):926-33.
5. Gerstein HC, Simpson JR, Atkinson S, Taylor DW, VanderMeulen J. The Diabetes Prevention Trial-Type 1 diabetes (DPT-1): implementation of screening and staging of relatives. DPT-1 Study Group. Feasibility and acceptability of a proposed infant feeding intervention trial for the prevention of type I diabetes. *Transplant Proc* 1995;27(6):3377.
6. Gale EA. Theory and practice of nicotinamide trials in pre-type 1 diabetes. *J Pediatr Endocrinol Metab* 1996;9(3):375-9.
7. Bingley PJ, Bonifacio E, Shattock M, Gillmor HA, Sawtell PA, Dunger DB, et al. Can islet cell antibodies predict IDDM in the general population? *Diabetes Care* 1993;16(1):45-50.
8. Hagopian WA, Sanjeevi CB, Kockum I, Landin-Olsson M, Karlsen AE, Sundkvist G, et al. Glutamate decarboxylase-, insulin-, and islet cell-antibodies and HLA typing to detect diabetes in a general population-based study of Swedish children. *J Clin Invest* 1995;95(4):1505-11.
9. Ongagna JC, Levy-Marchal C. Sensitivity at diagnosis of combined beta-cell autoantibodies in insulin-dependent diabetic children. French Registry of IDDM in Children Study Group. *Diabetes Metab* 1997;23(2):155-60.
10. Bonifacio E, Lampasona V, Genovese S, Ferrari M, Bosi E. Identification of protein tyrosine phosphatase-like IA2 (islet cell antigen 512) as the insulin-dependent diabetes-related 37/40K autoantigen and a target of islet-cell antibodies. *J Immunol* 1995;155(11):5419-26.
11. Gorus FK, Goubert P, Semakula C, Vandewalle CL, De Schepper J, Scheen A, et al. IA-2-autoantibodies complement GAD65-autoantibodies in new-onset IDDM patients and help predict impending diabetes in their siblings. The Belgian Diabetes Registry. *Diabetologia* 1997;40(1):95-9.
12. Bonifacio E, Genovese S, Braghi S, Bazzigaluppi E, Lampasona V, Bingley PJ, et al. Islet autoantibody markers in IDDM: risk assessment strategies yielding high sensitivity. *Diabetologia* 1995;38(7):816-22.
13. Wiest-Ladenburger U, Hartmann R, Hartmann U, Berling K, Bohm BO, Richter W. Combined analysis and single-step detection of GAD65 and IA2 autoantibodies in IDDM can replace the histochemical islet cell antibody test. *Diabetes* 1997;46(4):565-71.
14. Christie MR, Roll U, Payton MA, Hatfield EC, Ziegler AG. Validity of screening for individuals at risk for type I diabetes by combined analysis of antibodies to recombinant proteins. *Diabetes Care* 1997;20(6):965-70.
15. Kulmala P, Savola K, Petersen JS, Vahasalo P, Karjalainen J, Lopponen T, et al. Prediction of insulin-dependent diabetes mellitus in siblings of children with diabetes. A population-based study. The Childhood Diabetes in Finland Study Group. *J Clin Invest* 1998;101(2):327-36.
16. Schmidli RS, Colman PG, Bonifacio E. Disease sensitivity and specificity of 52 assays for glutamic acid decarboxylase antibodies. The Second International GADAB Workshop. *Diabetes* 1995;44(6):636-40.

17. Dittler J, Seidel D, Schenker M, Ziegler AG. GADIA2-combi Determination as First-Line Screening for Improved Prediction of Type 1 Diabetes in Relatives. *Diabetes* 1998;47(4):592-97.
18. Bingley PJ, Bonifacio E, Williams AJ, Genovese S, Bottazzo GF, Gale EA. Prediction of IDDM in the general population: strategies based on combinations of autoantibody markers. *Diabetes* 1997;46(11):1701-10.
19. Gardner SG, Bingley PJ, Sawtell PA, Weeks S, Gale EA. Rising incidence of insulin dependent diabetes in children aged under 5 years in the Oxford region: time trend analysis. The Bart's-Oxford Study Group [see comments]. *Brit Med J* 1997;315(7110):713-7.
20. Ruwaard D, Hirasing RA, Reeser HM, van Buuren S, Bakker K, Heine RJ, et al. Increasing incidence of type I diabetes in The Netherlands. The second nationwide study among children under 20 years of age. *Diabetes Care* 1994;17(6):599-601.
21. De Man SA, Grobbee DE, Hofman A. A study on bloodpressure and physical fitness in The Netherlands. *J Hypertension* 1989;7:429.
22. Petersen JS, Hejnaes KR, Moody A, Karlsen AE, Marshall MO, Hoier-Madsen M, et al. Detection of GAD65 antibodies in diabetes and other autoimmune diseases using a simple radioligand assay. *Diabetes* 1994;43(3):459-67.
23. Payton M, Hawkes C, Christie M. Relationship of the 37,000- and 40,000-M(r) tryptic fragments of islet antigens in insulin-dependent diabetes to the protein tyrosine phosphatase-like molecule IA-2 (ICA512). *J Clin Invest.* 1995;96(3):1506-1511.
24. Baekkeskov S, Aanstoot HJ, Christgau S, Reetz A, Solimena M, Cascalho M, et al. Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. *Nature* 1990;347(6289):151-6.
25. Marner B, Lernmark A, Nerup J, Molenaar JL, Tuk CW, Bruining GJ. Analysis of islet cell antibodies on frozen sections of human pancreas. *Diabetologia* 1983;25(2):93-6.
26. Batstra MR, Bruining GJ, Aanstoot HJ. Antibody screening in a population of children. *Ann Med* 1997;29(5):453-60.
27. Ronningen KS. Genetics in the prediction of insulin-dependent diabetes mellitus: from theory to practice. *Ann Med* 1997;29(5):387-92.
28. Grubin CE, Daniels T, Toivola B, Landin-Olsson M, Hagopian WA, Li L, et al. A novel radioligand binding assay to determine diagnostic accuracy of isoform-specific glutamic acid decarboxylase antibodies in childhood IDDM. *Diabetologia* 1994;37(4):344-50.
29. Jury KM, Loeffler D, Eiermann TH, Ziegler B, Boehm BO, Richter W. Evidence for somatic mutation and affinity maturation of diabetes associated human autoantibodies to glutamate decarboxylase. *J Autoimmun* 1996;9(3):371-377.
30. Richter W, Jury K, Loeffler D, Manfras B, Eiermann T, Boehm B. Immunoglobulin variable gene analysis of human autoantibodies reveals antigen-driven immune response to glutamate decarboxylase in type 1 diabetes mellitus. *Eur J Immunol.* 1995;25(6):1703-12.
31. Baekkeskov S, Warnock G, Christie M, Rajotte RV, Larsen PM, Fey S. Revelation of specificity of 64K autoantibodies in IDDM serums by high-resolution 2-D gel electrophoresis. Unambiguous identification of 64K target antigen. *Diabetes* 1989;38(9):1133-41.

Chapter 3.3

Low Prevalence of Antibodies to GAD65 in a 50 – 74-year-old General Dutch Population

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Running title: GAD65 antibodies in a general Dutch Population

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Low Prevalence of Antibodies to GAD65 in a 50- to 74-Year-Old General Dutch Population

The Hoorn Study

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OBJECTIVE — To assess the prevalence of antibodies to GAD65 (GAD65-A) in relation to glucose tolerance disturbances and to blood glucose-lowering therapy in a general Dutch population.

RESEARCH DESIGN AND METHODS — A population sample of 2,350 Dutch subjects, age 50–74 years, agreed to undergo an oral glucose tolerance test (OGTT). They were classified as having normal glucose tolerance, impaired glucose tolerance, newly detected diabetes, or known diabetes. GAD65-A levels were measured in serum by means of a standardized radioligand assay and subsequently were expressed as indexes. The prevalence rates were defined as the proportions of individuals of each category of glucose tolerance exceeding the value of the index at the 99th percentile of the entire study population.

RESULTS — The prevalence rates and the 95% CIs of GAD65-A were 0.7% (0.4–1.2%) in cases of normal glucose tolerance, 2.4% (0.9–5.3%) in impaired glucose tolerance, 0% (0–3.3%) in newly detected diabetes, according to the World Health Oganization (WHO) criteria, and 3.5% (0.7–10.0%) in known diabetes. A total of 2 out of 3 subjects with GAD65-A indexes above the 99th percentile and 10 out of 18 subjects with GAD65-A indexes above the 85th percentile received insulin therapy for their diabetes, which showed an association between GAD65-A and insulin therapy.

CONCLUSIONS — Low prevalence rates of latent autoimmunity to GAD were found in 50- to 74-year-old Dutch subjects with normal and abnormal glucose tolerance, and GAD65-A was associated with insulin use in known diabetic subjects.

Diabetes in adults is a heterogeneous disorder, with variation in clinical presentation. The majority of these patients present with characteristic features associated with insulin resistance and are classified as having NIDDM. A minority of patients present with features that are characteristic for neither NIDDM nor IDDM (1). Some have autoantibodies directed against the β-cell proteins of the pancreas, suggesting an autoimmune nature. In these patients, antibodies to GAD (GAD65-A) are specifically associated with difficulties in blood glucose regulation, which result in insulin therapy varying from a few weeks to a few years after clinical diagnosis (2,3). These patients were considered to have latent autoimmune diabetes (1,4). To establish the validity of GAD65-A in the prediction of the clinical course of adult-onset diabetes, a cross-sectional study was performed in which we measured GAD65-A in relation to glucose tolerance disturbances and to blood glucose–lowering diabetes therapy.

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CPM, counts per minute; GAD65-A, antibodies to the 65kD isoform of glutamic acid decarboxylase; OGTT, oral glucose tolerance test; WHO, World Health Organization.

RESEARCH DESIGN AND METHODS

Subjects

A random sample of 50- to 74-year-old subjects was taken from the population register of the town of Hoorn in the Netherlands (57,000 inhabitants). Of the 3,553 subjects invited, 2,540 (71.5%) participated, of whom 56 non-Caucasian subjects were excluded. Caucasian ethnicity was defined as having at least three grandparents from European or Mediterranean countries. The actual cohort therefore consisted of 2,484 subjects (5).

GAD65-A

Antibodies to the 65kD isoform of GAD were measured in serum and stored at −70°C by means of a radioligand assay as previously described (6,7). GAD65-A levels were expressed as index values measured in counts per minute (cpm). GAD65-A index = 1 + (cpm [unknown sample] − cpm [negative standard serum]) / (cpm [positive standard serum] − cpm [negative standard serum]). A constant of 1 was added to each index to facilitate interpretation, but this had no effect on the statistical tests. GAD65-A indexes showed positively skewed distributions as well as a minimum value for the general population of 0.9 and a maximum value of 5.8. GAD65-A indexes above the 99th percentile were arbitrarily defined as positive (6). Samples were tested three times and the mean value of these tests was used in the analysis. The interassay coefficient of variation was 7.5% (n = 72).

Oral glucose tolerance tests (OGTTs)

All subjects who were not treated with sulfonylurea, metformin, or insulin underwent a 75-g oral glucose tolerance test and were

Table 1—*GAD65-A indexes according to glucose tolerance category in a general Dutch population sample*

GAD65-A levels	Normal glucose tolerance	Impaired glucose tolerance	Newly detected diabetes	Known diabetes
n	1,909	245	111	85
>99.25th percentile	10 (0.5)	4 (1.6)	—	3 (3.5)†
>99th percentile*	14 (0.7)	6 (2.4)†	—	3 (3.5)†
>95th percentile	90 (4.7)	12 (4.9)	5 (4.5)	12 (14.1)†
>85th percentile	285 (14.9)	37 (15.1)	13 (11.7)	20 (23.5)†
Median (interquartile range)	0.977 (0.968–0.987)	0.976 (0.965–0.987)	0.976 (0.976–0.985)	0.981 (0.970–0.992)†

Data are *n* (% of corresponding category) or medians (interquartile range). GAD65-A levels are expressed as index values. *Approximate mean + 2 SD, GAD65-A indices above this value are defined as positive; †$P < 0.05$ vs. normal glucose tolerance.

classified according to the World Health Organization (WHO) (8) criteria as having normal glucose tolerance, impaired glucose tolerance, or newly detected diabetes. Verified known diabetes was defined as previously diagnosed diabetes treated with sulfonylurea, metformin, insulin, or diet-only, if the results of an OGTT met the WHO criteria for diabetes. The venous plasma glucose values were determined according to a glucose dehydrogenase method (Merck, Darmstadt, Germany). In 118 (4.8%) subjects, the GAD65-A could not be measured because of lack of serum, and in 16 subjects a glucose tolerance category could not be established because of an incomplete OGTT, resulting in 2,350 subjects being included in the analysis.

Biometry

BMI was calculated as weight (kilograms) divided by height (meters) squared.

Analysis

The differences in continuous variables between two groups were analyzed by means of the Student's *t* test for unpaired samples or the Mann-Whitney *U* test, when appropriate. A χ^2 test or Fisher's exact test was used to analyze differences between the groups in frequency data. All data were analyzed with an SPSS-PC software package, version 5.0 (SPSS, Chicago, IL), or Epitable (Coulombier, Charenton, France).

RESULTS — The prevalence rates and 95% CIs of antibodies to GAD65 were 0.7% (0.4–1.2%) in cases of normal glucose tolerance, 2.4% (0.9–5.3%) in impaired glucose tolerance, 0% (0–3.3%) in newly detected diabetes, and 3.5% (0.7–10.0%) in known diabetes. In each glucose tolerance category, GAD65-A indexes showed distributions that were positively skewed (distributions not shown). These distributions were similar for normal glucose tolerance, impaired glucose tolerance, and newly detected diabetes (Table 1). The distribution of GAD65-A indexes in known diabetes was significantly different from that in normal glucose tolerance. This difference is likely due to the GAD65-A indexes of subjects with known diabetes who were on insulin therapy: 10 out of 18 had GAD65-A indexes above the 85th percentile (Table 2). In addition, 3 subjects with known diabetes had GAD65-A indexes above the 99th percentile and were thus considered to be positive. Two of these three subjects, aged 50 and 54 years, were on insulin therapy (ages at onset of diabetes were 35 and 49 years). The third was 68 years old and was only on a diet (age at onset was 44 years). The mean BMI of the 23 subjects in the entire study population who had GAD65-A was 7.5% below the mean BMI of the remaining subjects without GAD65-A (Table 3). The mean age did not differ, and 70% of those with GAD65-A were female.

CONCLUSIONS — We found a marginal increase in prevalence rates of GAD65-A in known diabetes and impaired glucose tolerance compared with normal glucose tolerance and no increase in prevalence rates in newly detected diabetes. The increase in known diabetes was less evident than previously suggested (1,4,9). The skewness of the distributions of GAD65-A indexes convinced us to use percentiles rather than standard deviations to define

Table 2—*Characteristics of subjects with known diabetes according to blood glucose–lowering therapy*

	Diet only	Sulfonylurea and/or metformin	Insulin
n	12	55	18
GAD65-A index			
>99.25th percentile	1 (8.3)	—	2 (11.1)
>99th percentile*	1 (8.3)	—	2 (11.1)
>95th percentile	2 (16.7)	4 (7.3)†	6 (33.3)
>85th percentile	2 (16.7)	8 (14.5)†	10 (55.6)
Other characteristics			
Sex (F/M)	8/4	32/23	10/8
Age (years)	65 ± 4.7	66.2 ± 6.5	62.7 ± 6.5
BMI (kg/m²)	24.8 ± 8.4	29.3 ± 5.2	27.4 ± 4.2
Fasting plasma glucose (mmol/l)	9.6 ± 2.9†	10.3 ± 3.5†	12.7 ± 3.7
Duration of diabetes (years)	11.1 ± 19.2	9.7 ± 12.4	15.4 ± 16.2
Onset of diabetes (years)	54.5 ± 19.6	56.6 ± 14.5†	47.3 ± 15.1

Data are means ± SD or *n* (% of corresponding category). *Approximate mean + 2 SD, GAD65-A indexes above this value are defined as positive; †$P < 0.05$ vs. insulin therapy.

Table 3—*Characteristics of the study population in relation to the presence or absence of antibodies to GAD65-A*

	GAD65-A	
	Presence†	Absence
n	23	2,327
Sex (F/M)	16/7	1,236/1,091
Age (years)	62.2 ± 7.8	61.7 ± 7.4
BMI (kg/m²)	24.5 ± 6.4*	26.5 ± 3.5

Data are *n* or means ± SD. *$P = 0.008$ vs. absence of GAD65-A; †GAD65-A indexes above the 99th percentile.

positivity, using a threshold for GAD65-A at the 99th percentile (index value 1.2). This almost coincides with the mean of the population + 2 SD (index value 1.4). A recent report with follow-up data on GAD65-A–positive diabetes patients suggested that only high levels of GAD-antibodies predict the need for insulin treatment (10). We considered several thresholds and tested all samples again with an index in the high range of the radioligand assay by means of a classical immunoprecipitation assay, evaluated by SDS-PAGE (11). The present data retrieved from a general population sample showed an overall prevalence rate of GAD65-A, similar to that found in an earlier Dutch general population study (12), and a rate of 3.5% for the category of known diabetes. In this category, the association between high GAD65-A indexes and insulin therapy suggests ongoing β-cell destruction. However, the prevalence rate is low compared to the findings of other studies in adult-onset diabetes. These studies have reported varying rates, possibly because of ethnic differences or the use of different criteria for the selection of study subjects. Prevalence rates were reported to be 4.3% in Japan (13), 1.7% in Korea (14), and 1.1% in Papua New Guinea (15), while for white subjects, adult rates between 3 and 36% have been reported (9,16,17). Other explanations for these discrepancies could be differences in applied assays for GAD antibodies and threshold selection. It is also possible that IDDM subjects in the Netherlands develop the disease at a younger age, compared with a population with a higher prevalence rate of GAD65A. A high proportion of insulin-requiring diabetic subjects showed GAD65-A indexes above the 85th percentile (Table 2). The most likely explanation for that intriguing phenomenon is that these diabetic subjects once were positive (GAD65A indexes above the 99th percentile) and that the duration of the disease resulted in a decreased level. No explanation has been found for the association between a low BMI and GAD65-A positivity in the population. We used GAD antibodies instead of other diabetes-specific antibody markers, since it seems to be the most sensitive marker in older persons (18). We conclude that the prevalence of GAD65-A in Dutch Caucasian subjects is low in any category of glucose tolerance and that distributions of GAD65-A indexes were similar for normal glucose tolerance, impaired glucose tolerance, and newly detected diabetes. Although this cross-sectional study does not allow definite conclusions, an important role for GAD65-A as a predictive marker for insulin dependency in Dutch subjects who present with diabetes after the age of 50 appears to be very unlikely. The results also suggest a low frequency of latent autoimmunity in such a population.

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References

1. Zimmet PZ: The pathogenesis and prevention of diabetes in adults: genes, autoimmunity, and demography. *Diabetes Care* 18:1050–1064, 1995
2. Hagopian WA, Karlsen AE, Gottsäter A, Landin-Olsson M, Grubin CE, Sundkvist G, Petersen JS, Boel E, Dyrberg T, Lernmark A: Quantitative assay using recombinant human islet glutamic acid decarbocylase (GAD65) shows that 64K autoantibody positivity at onset predicts diabetes type. *J Clin Invest* 91:368–374, 1993
3. Niskanen LK, Tuomi T, Karjalainen JK, Groop LC, Uusitupa MIJ: GAD antibodies in NIDDM: ten-year follow-up from the diagnosis. *Diabetes Care* 18:1557–1565, 1995
4. Zimmet PZ, Tuomi T, Mackay IR, Rowley MJ, Knowles W, Cohen M, Lang DA: Latent autoimmune diabetes mellitus in adults (LADA): the role of antibodies to glutamic acid decarboxylase in diagnosis and prediction of insulin dependency. *Diabetic Medicine* 11:299–303, 1994
5. Mooy JM, Grootenhuis PA, de Vries H, Valkenburg HA, Bouter LM, Kostense PJ, Heine RJ: Prevalence and determinants of glucose intolerance in a Dutch Caucasian population. *Diabetes Care* 18:1270–1273, 1995
6. Petersen JS, Hejnaes KR, Moody A, Karlsen AE, Marshall MO, Hoier-Madsen M, Boel E, Michelsen BK, Dyrberg T: Detection of GAD65 antibodies in diabetes and other autoimmune diseases using a simple radioligand assay. *Diabetes* 43:459–467, 1994
7. Schmidli RS, Colman PG, Bonifacio E: Disease sensitivity and specificity of 52 assays for glutamic acid decarboxylase antibodies: the Second International GADAB Workshop. *Diabetes* 44:636–640, 1995
8. World Health Organization: *Diabetes Mellitus: Report of a WHO Study Group*. Geneva, World Health Org., 1985 (Tech. Rep. Ser., no. 727)
9. Rowley MJ, Mackay IR, Chen QY, Knowles WJ, Zimmet PZ: Antibodies to glutamic acid decarboxylase discriminate major types of diabetes mellitus. *Diabetes* 41:548–551, 1992
10. Seubert M, de Sonnaville JJJ, Moser L, Seissler J, Heine RJ: Prevalence and predictive value of GAD-antibodies in diabetes mellitus diagnosed above 40 years of age. *Diabetologia* 39 (Suppl. 1):A91–A338, 1996
11. Aanstoot HJ, Kang SM, Kim J, Lindsay L, Roll U, Knip M, Atkinson M, Mose-Larsen P, Fey S, Ludvigsson J, Landin M, Bruining J, Maclaren N, Akerblom HK, Baekkeskov S: Identification and characterization of glima 38, a glycosylated islet cell membrane antigen, which together with GAD65 and IA2 marks the early phases of autoimmune response in type 1 diabetes. *J Clin Invest* 97:2772–2783, 1996
12. Aanstoot HJ, Petersen JS, Batstra MR, de Man S, Scholtens A, Jensen E, Molenaar JL, de Bruijn T, Grobbee D, Bruining GJ, Dryberg T: Low frequency of antibodies to glutamic acid decarboxylase in the general population. *Diabetologia* 38 (Suppl. 1): A54–A205, 1995
13. Tsuruoka A, Matsuba I, Toyota T, Isshiki G, Nagataki S, Ikeda Y: Antibodies to GAD in Japanese diabetic patients: a multicenter study. *Diabetes Res Clin Pract* 28:191–199, 1995
14. Park Y, Lee H, Koh CS, Min H, Rowley M, Mackay IR, Zimmet P, McCarthy B, McCanlies E, Dorman J, Trucco M: The low prevalence of immunogenetic markers in Korean adult-onset IDDM patients. *Diabetes Care* 19:241–245, 1996
15. Dowse GK, Zimmet PZ, Spark RA, Mavo B, Rowley MJ, Mackay IR: Lack of antibodies to glutamic acid decarboxylase in young adults of the high diabetes prevalence Wanigela people of Papua New Guinea. *Diabetes Res Clin Pract* 24:195–198, 1994
16. Zimmet PZ, Shaten BJ, Kuller LH, Rowley MJ, Knowles WJ, Mackay IR: Antibodies to glutamic acid decarboxylase and diabetes mellitus in the Multiple Risk Factor Intervention Trial. *Am J Epidemiol* 140:683–690, 1994
17. Tuomilehto J, Zimmet P, Mackay IR, Koskela P, Vidgren G, Toivanen L, Tuomilehto-Wolf E, Kohtamaki K, Stengard J, Rowley MJ: Antibodies to glutamic acid decarboxylase as predictors of insulin-dependent diabetes mellitus before clinical onset of disease. *Lancet* 343:1383–1385, 1994
18. Pietropaolo M, Hutton JC, Eisenbarth GS: Protein tyrosine phosphatase–like proteins: link with IDDM. *Diabetes Care* 20:208–214, 1997

Chapter 4

Factors affecting sensitivity and specificity in ß-cell antibody screening

Chapter 4.1

GAD-antibodies in screening for autoimmune diabetes – influence of co-morbidity and age and sex on specificity and threshold levels

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Submitted

4.1.1. Abstract

4.1.1.1. Background

Antibodies against glutamic acid decarboxylase (GAD_{65}) can be applied to predict or diagnose type·1 diabetes mellitus. In the future this application might have therapeutic consequences. Therefore it is important to define factors that influence GAD·antibody screening specificity.

4.1.1.2. Methods

We evaluated the impact of age and sex on GAD·antibody levels in 1287 individuals from the general population and analyzed how adjustment of thresholds changed screening specificity. In addition, we studied whether GAD·antibody frequencies are increased in sera (n = 623) of patients with diseases involving tissues in which GAD is expressed (epilepsy, cystic fibrosis, Guillain Barré syndrome and premature ovarian failure).

4.1.1.3. Results & conclusions

GAD·antibody levels were slightly correlated to age and sex but threshold adjustment did not significantly alter screening specificity.

This finding justifies the use of assay thresholds derived from age and sex non-defined populations. In addition, diseases involving tissues where GAD_{65} is expressed did not result in increased GAD·antibody frequencies.

4.1.2. Introduction

Type·1 diabetes mellitus is a T·cell mediated autoimmune disease characterized by progressive destruction of the insulin producing ß·cell. At onset of type·1 diabetes islet cell antibodies (ICAs), antibodies against the 65 kilodalton isoform of glutamic acid decarboxylase (GAD_{65}) and the tyrosine phosphatase IA2, are detectable in 80 – 95% of the patients [1, 2]. Autoantibodies can be detected several years before clinical onset and can

be used to predict the development of type-1 diabetes mellitus [1, 3-7]. At present, autoantibody tests are used as an inclusion criterion in several diabetes prevention trials [8, 9].

Evidence is accumulating that type-1 diabetes may become clinically manifest in adults initially diagnosed as having type-2 diabetes - a condition that is often referred to as latent autoimmune diabetes in adults (LADA). In these patients a rapid decline in ß-cell function and onset of insulin dependency is better correlates to the presence of GAD-antibodies than to other clinical or biochemical parameters (BMI, C-peptide, HBA1c) at onset of the disease. Antibodies can thus be used as a diagnostic tool for type-1 diabetes in adults [10-12]. Early discrimination between type-1 and type-2 diabetes is important to guide glucoregulatory therapy and may allow early application of preventive strategies in patients otherwise not considered to have type-1 diabetes. In addition, classification is important for research purposes.

Thus, GAD-antibodies can be used both as a predictive as well as a diagnostic tool for type-1 diabetes. However, sensitivity and specificity of GAD-antibody screening have not been fully characterized and the specificity of screening varies from 20 to 70% in different populations [2, 3, 7, 10, 12-15]. When screening has therapeutic consequences this means that 30-80% will be unnecessarily treated. Since any study or therapy initiated carries the risk of unwanted dangerous side effects, it is extremely important to minimize this number. The current study aims to identify causes of false positivity in GAD-antibody screening for type-1 diabetes.

Specificity of screening is strongly dependent on the definition of thresholds [16, 17]. Thresholds for positivity for GAD-antibodies have generally been defined in populations of children since the primary aim has been prediction of type-1 diabetes. These thresholds might not be applicable when testing for type-1 diabetes in adults.

Previously, Petersen and colleagues demonstrated that the frequency of GAD-antibodies in mixed connective tissue disease (MCTD) is increased compared to controls [18]. In addition, stiff man syndrome, a rare neurological disease, is associated with GAD-antibodies in the majority of patients but less than half of the patients develop type-1 diabetes [19]. Both observations demonstrate that there are conditions leading to the formation of GAD-antibodies but not to type-1 diabetes. MCTD is associated with diffuse cerebral dysfunction. We reasoned that involvement of the central nervous system might explain GAD-antibody formation in these patients. Leakage of GAD from brain into the circulation may lead to immune-activation and consequent formation of GAD-antibodies. GAD is expressed in islets of Langerhans, neuronal tissue, ovaries and testes [20, 21]. Co-morbidity involving these tissues may therefore have to be taken into account when screening individuals for GAD-antibodies for prediction or diagnosis of type-1 diabetes.

In the current study age and sex effects on GAD-antibody levels and the effect of threshold adjustment to age and sex on screening specificity were evaluated in a sample of 1287 individuals from the general population. In addition, the prevalence of GAD-antibodies in diseases involving pancreas, neuronal tissue or ovaria (Cystic fibrosis, epilepsy, Guillain Barré syndrome, and premature ovarian failure) was compared to the prevalence in the general population.

4.1.3. Materials and Methods

4.1.3.1. Populations studied

The demographic data of the populations are shown in table 4.1.1. The general population consisted of 1287 individuals from Zoetermeer, a town in the southwestern part of The Netherlands, who participated in a study of cardiovascular risk factors. Sera were collected in 1976 and stored at -20°C until testing. The population is described in detail elsewhere [22].

Five-hundred-and-twenty-two sera from 394 patients aged one month to 16 years who participated in the Dutch study of epilepsy in childhood [23, 24] were tested for GAD-antibodies. The participants of the current study were selected from a cohort of children with a first unprovoked seizure or newly diagnosed epilepsy, if the diagnosis was confirmed based on EEG or therapy. Sera were collected shortly after the index (presenting) seizure and at six months and one year duration of epilepsy. In the current study all sera collected within two months (mean 0.7 months) after the index seizure (n=228) and sera collected at the longest disease duration available of each patient (mean 12.2 months, range 2 – 50 months) (n=294) were analyzed separately. The diagnosis and development of diabetes during follow-up (five years) were recorded from the medical records.

Forty-three sera from 38 cystic fibrosis patients from the Sophia Children's Hospital were tested for GAD.antibodies. The patients participated in a study on the pharmacodynamics of antibiotics (tested in 1990-1992). Samples were frozen at -80°C after collection and never thawed before the GAD-antibody analysis.

Sera from 30 patients with premature ovarian failure and 28 sera from patients with Guillain Barré Syndrome (14m, age range 19-64) were tested for GAD-antibodies. Sera were collected from 1990 to 1992 and stored at -80°C. The study protocols were approved by the appropriate medical ethical committees.

4.1.3.2. GAD-antibody assays

Sera were tested for GAD-antibodies by radio binding assay (RBA) [16] or immunoprecipitation and SDS-PAGE and evaluated using autoradiography (conventional immunoprecipitation: cIMP) [25] as indicated in table 4.4.1. For the RBA human recombinant ^{35}S methionine labeled GAD_{65} was produced in an in vitro transcription translation system (Promega Madison, WI, USA). Free methionine was removed by gel-filtration on a

NAP5 column (Amersham Pharmacia Biotech, Uppsala, Sweden). Immune complexes were precipitated using 10 µg Protein A Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden) and washing was performed in a Multiscreen system (Millipore, Bedford, Ma, USA). Precipitated radioactivity was counted in a microbeta plate reader (EG&G Wallac, Wellesley, Ma, USA). All sera were analyzed in triplicate. Internal reference sera were included in each plate for epilepsy and cystic fibrosis patients and in each third plate for the general population. Using the internal reference sera, an index (GAD-index) was calculated for the purpose of comparison of experiments [18]. The GAD-antibody concentration of the positive reference serum was in the linear range of the dilution curve, which allows the GAD-index to be interpreted semi-quantitatively. The threshold for positivity was defined as the 99.5th centile of a population of 1403 schoolchildren as described before (GAD-index >0.21) [16]. To study whether threshold adjustment to age improves screening specificity, we analyzed the autoantibody frequency in the general population applying the 99.5th centile of the general population as a whole and of 10 equally sized age groups as a threshold.

The cIMP was performed according to the protocol described by Baekkeskov and collegues, using ^{35}S methionine labeled fetal rat islets (a gift from Dr. T. Dyrberg and H Richter-Olesen, The Hagedorn research institute, Gentofte, Denmark) [25].

4.1.3.3. Statistical evaluation

The statistical package SPSS for Windows (version 7.5.2; SPSS Inc., Chicago, IL, USA) was used for data analysis. Differences between groups were analyzed using the Chi square, Mann Whitney and Kruskal Wallis tests. Trends within groups were analyzed by the Spearman's correlation test.

Table 4.1.1. demographic data of populations tested for GAD antibodies.

Population:	General population	Epilepsy (all)	Epilepsy (onset)	Epilepsy (long duration)	Cystic Fibrosis	Premature Ovarian Failure	Guillain Barre Syndrome
N	1287	522	228	294	43	30	28
Age range (mean)	6 – 86 (32)	6-19 (6.42)	0-14 (5.77)	1 – 19 (6.87)	-	-	19 - 64
Disease duration (mean)	n.a.	0-50 Mo (6.9)	0–1.5 Mo (0.23)	2-50 Mo (12)	-	-	-
Median GAD index (range)	0.04 (-0.07 – 1.71)	0.01* (-0.1 – 1.45)	0.01* (-0.1 – 1.45)	0.02* (-0.1 – 0.28)	-0.05* (-0.08 – 0.21)	-	-
% positive RBA (n)	1.0% (13)	0.8% (4)	0.9% (2)	0.6% (2)	2.3% (1)	n.t.	n.t.
n positive c IMP	n.t.	4	2	2	n.t.	0	1
CIMP / RBA	RBA	RBA/ cIMP	RBA/ cIMP	RBA/ cIMP	RBA	cIMP	cIMP

* p = <0.001 compared to general population

Table 4.1.2. Antibody frequency in the general population applying thresholds adjusted to age and a general threshold

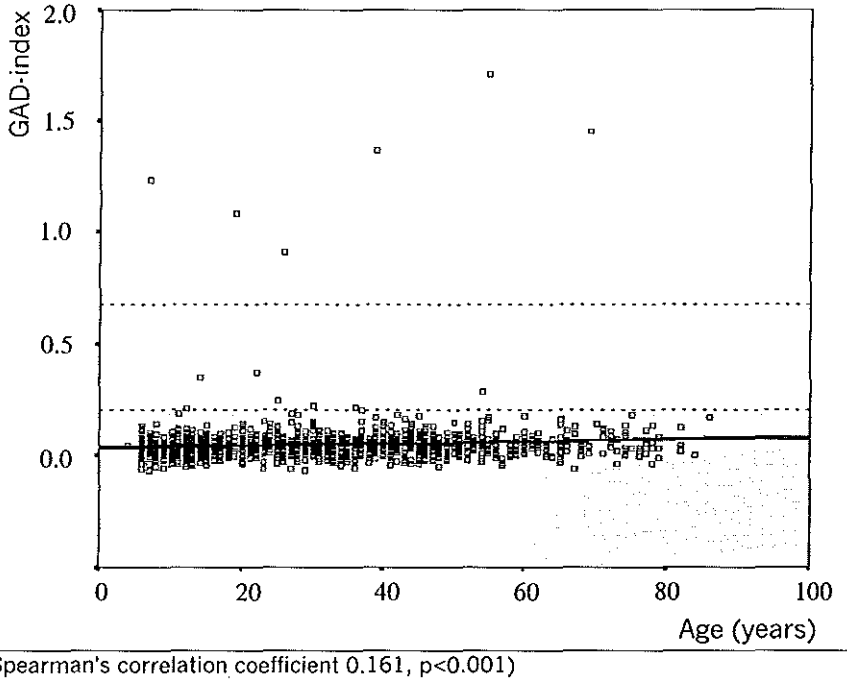
Age:	<9	9 - 13	13 - 17	17 - 23	23-29	29 - 35	35 - 41	41- 47	47 - 56	>56	all
n	101	147	136	117	129	131	127	125	140	134	**1287**
Mean GAD-idx	0.04	0.03	0.03	0.05	0.06	0.05	0.06	0.05	0.06	0.06	**0.05**
Median GAD-idx	0.03	0.03	0.03	0.03	0.05	0.04	0.05	0.05	0.04	0.04	**0.04**
Adjusted threshold	0.69	0.20	0.21	0.67	0.49	0.18	0.65	0.18	0.72	0.61	**0.68**
n > age adjusted threshold	1	1	1	1	1	1	0	1	1	1	**9**
n > population threshold(0.68)	1	0	0	1	1	0	1	0	1	1	**6**
n > 0.21 (original threshold)	1	1	1	2	2	1	2	0	2	1	**13**

4.1.4. Results

4.1.4.1. General population

The antibody frequency in the general population was 1% (table 4.1.1.). The positive individuals did not differ from the negative individuals in age or sex distribution.

Figure 4.1.1. Correlation between age and GAD-antibody levels in 1287 individuals from the general population



(Spearman's correlation coefficient 0.161, p<0.001)

Figure 4.1.1. represents a scatterplot of age versus GAD-index. There was a slight, but statistically significant, correlation between age and GAD-index (Spearman's correlation coefficient 0.161, p<0.001). In addition, the GAD-index in women was significantly higher than in men (p=0.009, median GAD-index 0.043 and 0.037, respectively). The correlation between sex and GAD-index did not explain the correlation with age and vice versa.

To study whether the observed correlation with age affects threshold definition, the general population was split in ten similarly sized age-groups (table 4.1.2.). The threshold for positivity was adjusted to the 99.5th centile of each age group and compared to the 99.5th centile threshold of the general population (0.68, table 4.1.2.). This yielded four additional positive individuals, one of these was negative at the initially applied threshold of 0.21. One individual who was positive at the general population threshold was negative when applying the age adjusted thresholds. Adjustment of thresholds to sex did not affect the antibody frequencies.

Strikingly, the GAD-index was significantly higher in the general population than in all other populations analyzed for this study (table 4.1.1.). We therefore compared the data from the general population to the data from a cohort of 1403 schoolchildren (age 10–12) that was previously analyzed [16]. The median GAD-index in the general population was increased compared to the schoolchildren. To exclude that the observed differences in GAD-index are due to age effects, we selected all individuals aged 10–12 from the general population and compared their GAD-index to the GAD-index in the schoolchildren cohort. The median GAD-index in this selection of the general population (0.04) was still significantly higher than in the schoolchildren cohort (-0.01). The data of the general population were therefore analyzed a second time, applying the 99.5th centile of the general population as a threshold for positivity (GAD-index 0.68 compared to 0.21 in the previous analysis). This yielded six (0.46%) instead of 13 positive individuals in the general population.

4.1.4.2. Analysis of co-morbidity

The antibody frequencies in the patient populations were compared to the frequencies in the general population and in the population of schoolchildren. The GAD-antibody frequency in 522 epilepsy sera (0.8%) was neither significantly increased compared to the general population nor

to the population of schoolchildren. The GAD-antibody frequency at onset did not differ from the frequency at long disease duration and positive individuals did not differ from the negative individuals in age distribution. Sera taken at onset and long duration did not significantly differ in GAD-index from either the population of schoolchildren or each other (table 4.1.1.). The antibody levels or frequency were not correlated to the duration of epilepsy.

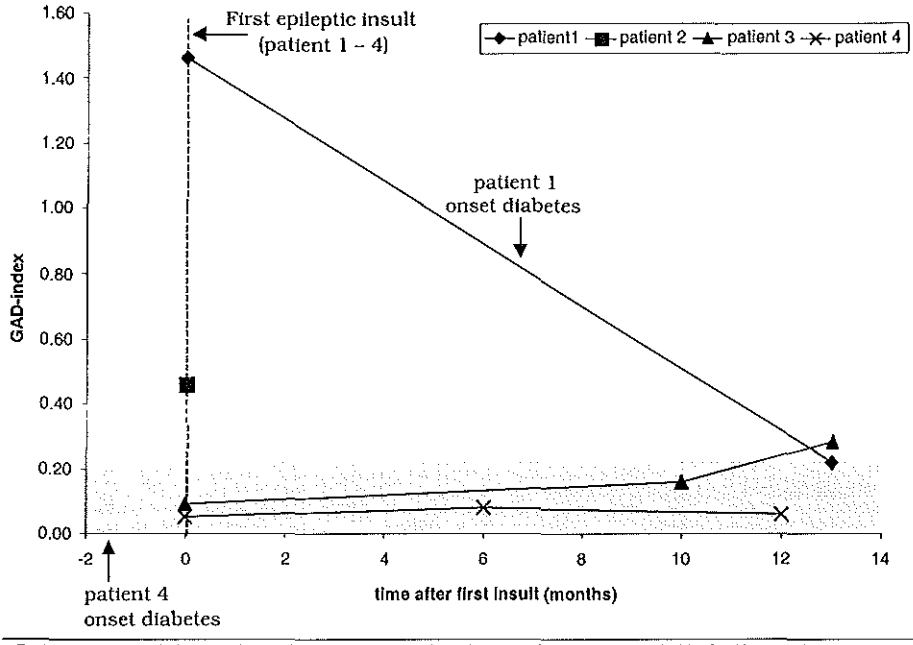
Four sera from three epilepsy patients were positive for GAD-antibodies. Two sera came from one patient (figure 4.1.2. - patient 1) who developed epilepsy at age 2 and diabetes at age 2½. The sera were drawn at onset (GAD-index 1.46) of epilepsy and 13 months later (GAD-index 0.22). Another GAD-antibody positive serum was taken at epilepsy onset from a patient at age 11 (figure 4.1.2. – patient 2, GAD-index 0.46). No additional sera were available from this patient. A third epilepsy patient (figure 4.1.2. – patient 3) was positive (GAD-index 0.28) at 13 months disease duration but negative in previous samples taken at onset (GAD-index 0.096) and 10 months after the first seizure (GAD-index 0.16). Patients 2 and 3 did not develop diabetes during five years follow-up. Of two epilepsy patients who had diabetes, only one patient was positive for GAD-antibodies (figure 4.1.2. – patient 1 and 4).

The median GAD-index in cystic fibrosis patients was low (-0.05) and significantly differed from the GAD-index in the general population, but not from the epilepsy patients or the population of schoolchildren. One of the cystic fibrosis patients had a GAD-index of 0.21, which is just at the defined threshold for positivity. A serum sample from this patient collected three months later was negative (GAD-index 0.16). Considering this patient

as positive did not result in a significant difference in GAD-antibody frequency between the general population and the cystic fibrosis patients.

None of 30 premature ovarian failure patients and one of 28 Guillain Barré syndrome patients were positive for GAD-antibodies.

Figure 4.1.2. Course of GAD-antibodies in 4 epilepsy patients.



Patients 1 and 4 developed diabetes at the time-point indicated. T=0: time of first epileptic seizure.

4.1.5. Conclusion and discussion

GAD-antibodies can be applied for prediction and diagnosis of type-1 diabetes. While prediction currently is still reserved for research purposes, the use of screening for GAD-antibodies is gaining popularity as a tool to discriminate between type-2 and type-1 diabetes. In the future both applications might have therapeutical consequences. Dependent on threshold definition the specificity of screening may be as low as 92.8%, which means that a substantial number of people will be unjustly treated [16]. The current study evaluates how threshold adjustment to age and sex and exclusion of co-morbidity involving GAD containing tissues may alter specificity of GAD-antibody screening for prediction and diagnosis of type-1 diabetes.

4.1.5.1. General population

We observed a slight, but statistically significant, positive correlation between age and GAD-index in the general population. Adjustment of thresholds to age resulted in highly variable thresholds per age group (table 4.1.2.). However, the median GAD-index per age group was not significantly different, indicating that the variation in thresholds is due to statistical variation, especially since these thresholds do not reflect the positive correlation with age. Thus, it is not necessary to use age-matched controls when establishing reference values for GAD-antibody screening. Although on the population level the effect of threshold adjustment to age or sex is limited, the impact of slight aberrations in threshold definition for the individual should not be underestimated (table 4.1.2.). Therefore, assay-thresholds need to be meticulously monitored.

In the general population the GAD-index was significantly increased compared to the patient populations studied. This is in contrast to what we expected based on the hypothesis of this study. Using sera from a population of schoolchildren we demonstrated that this observation was not due to age effects. Since the general population and the schoolchildren were tested in one experiment (using one batch of tracer and protein A Sepharose) the differences can neither be explained by technical variation nor by the methods employed. A noticeable difference between both populations was serum storage: The general population sera were stored at -20°C for 16 years in tubes with snap caps. When defrosted, some of these sera contained precipitates. The sera of the schoolchildren cohort, on the contrary, were stored at -80°C for 10 years in tubes with screw caps and had never been defrosted before GAD-antibodies were analyzed. It is likely that the storage conditions of the samples from the general population have resulted in the observed elevated antibody levels in the general population sera. This observation implicates that storage conditions should be carefully monitored in order to exclude such technical problems in future studies. Since all sera from the general

population were stored in one freezer in identical tubes for the same period of time, it is not likely that storage has affected the analysis of age and sex effects in the general population. In addition, positive samples did not differ from negative samples in macroscopic aspect.

4.1.5.2. Effect of co-morbidity on specificity of screening

GAD is not only expressed in the endocrine pancreas but in ovaria, testes and brain as well [20, 21]. Destruction of these tissues may result in leakage of GAD into the circulation, with consequent activation of the immune system and formation of GAD-antibodies. Since GAD-antibodies are not pathogenic, this is not necessarily associated with the development of type-1 diabetes mellitus. Studies of animal models have demonstrated that this may depend on genetic susceptibility of the immune system to autoimmunity [26]. We studied if such a mechanism occurs by comparing the frequency of GAD-antibodies in the general population to the frequency in a group of non-autoimmune and a group of autoimmune mediated diseases involving presumed destruction of tissues that express GAD.

Five-hundred-twenty-two sera from 394 patients with epilepsy and 43 sera from 38 cystic fibrosis patients were studied as example of non-autoimmune mediated diseases. The rationale behind this selection is that epileptic seizures may result in brain damage and consequent leakage of GAD into the circulation. Cystic fibrosis patients were selected because in these patients non-autoimmune mediated ß-cell destruction occurs. This has been demonstrated in autopsy studies [27] and is confirmed by the observation of Marner an colleagues, who reported that cystic fibrosis related diabetes is not associated with the formation of ICAs [28]. The pathogenesis of premature ovarian failure and Guillain Barré is still controversial but it is assumed that autoimmunity contributes to the pathogenesis. Since both diseases involve GAD-expressing tissues (ovaria and nervous tissue, respectively), they were evaluated in the current study.

The antibody frequency in the patient populations was not increased compared to the general population or the population of schoolchildren. These observations are in line with the observation of Marner and colleagues, that pancreas fibrosis does not lead to the formation of ICAs, regardless the development of glucose intolerance or overt diabetes [28].

One might argue that the employment of different methods for antibody detection in the patients and reference populations invalidates the comparative analysis. However, in a previous study [16] and in the epilepsy patients (table 4.1.1.) we demonstrated that the 99.5th centile threshold of the RBA coincides with the detection limit of the conventional immunoprecipitation. Furthermore, if there is a difference in sensitivity between the RBA and the conventional immunoprecipitation, the latter is likely to have higher sensitivity, since it is characterized by a lower aspecific background, due to the fact that GAD is unambiguously identified on the fluorogram. In the current study we hypothesized that the GAD-antibody frequency in neuroendocrine patients was higher, due to non-diabetes associated formation of GAD-antibodies. Application of the highly sensitive immunoprecipitation technique in the patient populations would only result in exaggeration of this effect. The fact that we did not observe any statistical significant increase in the antibody frequency in the patient populations justifies the conclusion that premature ovarian failure and Guillain Barré syndrome do not result in increased GAD-antibody formation. However, one out of 28 (3.6%) Guillain Barré syndrome patients was positive for GAD-antibodies. Although this is not significantly more than in the general population or the schoolchildren cohort, an additional study is needed to draw definite conclusions on the formation of GAD-antibodies in Guillain Barré syndrome.

Thus, the current study did not provide evidence for the hypothesis that the formation of GAD-antibodies may be initiated by leakage of GAD from damaged tissue into the circulation, neither in non-autoimmune nor in autoimmune mediated diseases. Therefore such conditions need not be

taken into account when screening for type-1 diabetes. It is conceivable that other factors, such as defective apoptosis, aberrant presentation to the immune system or a defect in the immune system are necessary to induce autoimmunity to GAD.

4.1.5.3. Conclusion

This study demonstrates that for definition of reference values for GAD-antibodies age and sex non-defined populations can be used. In addition we have demonstrated that co-morbidity involving degeneration of tissues that express GAD needs not be taken into account when screening for GAD-antibodies. International agreement on standardization using WHO standard sera and threshold definition are needed to explain differences between specificity of screening in different populations. Since storage conditions may significantly affect the outcome of GAD-antibody tests these need to be meticulously monitored in collaborative studies.

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References

1. Aanstoot HJ, Kang SM, Kim J, Lindsay LA, Roll U, Knip M, et al. Identification and characterization of glima 38, a glycosylated islet cell membrane antigen, which together with GAD65 and IA2 marks the early phases of autoimmune response in type 1 diabetes. *J Clin Invest* 1996;97(12):2772-83.
2. Borg H, Fernlund P, Sundkvist G. Protein tyrosine phosphatase-like protein IA2-antibodies plus glutamic acid decarboxylase 65 antibodies (GADA) indicates autoimmunity as frequently as islet cell antibodies assay in children with recently diagnosed diabetes mellitus. *Clin Chem* 1997;43(12):2358-63.
3. Batstra MR, Bruining GJ, Aanstoot HJ. Antibody screening in a population of children. *Ann Med* 1997;29(5):453-60.
4. Bingley PJ, Bonifacio E, Williams AJ, Genovese S, Bottazzo GF, Gale EA. Prediction of IDDM in the general population: strategies based on combinations of autoantibody markers. *Diabetes* 1997;46(11):1701-10.
5. Wiest-Ladenburger U, Hartmann R, Hartmann U, Berling K, Bohm BO, Richter W. Combined analysis and single-step detection of GAD65 and IA2 autoantibodies in IDDM can replace the histochemical islet cell antibody test. *Diabetes* 1997;46(4):565-71.
6. Kulmala P, Savola K, Petersen JS, Vahasalo P, Karjalainen J, Lopponen T, et al. Prediction of insulin-dependent diabetes mellitus in siblings of children with diabetes. A population-based study. The Childhood Diabetes in Finland Study Group. *J Clin Invest* 1998;101(2):327-36.
7. Roll U, Ziegler AG. Combined antibody screening for improved prediction of IDDM-- modern strategies. *Exp Clin Endocrinol Diabetes* 1997;105(1):1-14.
8. Gale EA. Theory and practice of nicotinamide trials in pre-type 1 diabetes. *J Pediatr Endocrinol Metab* 1996;9(3):375-9.
9. Gerstein HC, Simpson JR, Atkinson S, Taylor DW, VanderMeulen J. The Diabetes Prevention Trial-Type 1 diabetes (DPT-1): implementation of screening and staging of relatives. DPT-1 Study Group. Feasibility and acceptability of a proposed infant feeding intervention trial for the prevention of type I diabetes. *Transplant Proc* 1995;27(6):3377.
10. Seissler J, de Sonnaville JJ, Morgenthaler NG, Steinbrenner H, Glawe D, Khoo-Morgenthaler UY, et al. Immunological heterogeneity in type I diabetes: presence of distinct autoantibody patterns in patients with acute onset and slowly progressive disease. *Diabetologia* 1998;41(8):891-7.
11. Hagopian WA, Karlsen AE, Gottsater A, Landin-Olsson M, Grubin CE, Sundkvist G, et al. Quantitative assay using recombinant human islet glutamic acid decarboxylase (GAD65) shows that 64K autoantibody positivity at onset predicts diabetes type. *J Clin Invest* 1993;91(1):368-74.
12. Zimmet PZ, Tuomi T, Mackay IR, Rowley MJ, Knowles W, Cohen M, et al. Latent autoimmune diabetes mellitus in adults (LADA): the role of antibodies to glutamic acid decarboxylase in diagnosis and prediction of insulin dependency. *Diabet Med* 1994;11(3):299-303.
13. Bingley PJ, Bonifacio E, Shattock M, Gillmor HA, Sawtell PA, Dunger DB, et al. Can islet cell antibodies predict IDDM in the general population? *Diabetes Care* 1993;16(1):45-50.
14. Tuomi T, Groop LC, Zimmet PZ, Rowley MJ, Knowles W, Mackay IR. Antibodies to glutamic acid decarboxylase reveal latent autoimmune diabetes mellitus in adults with a non-insulin-dependent onset of disease. *Diabetes* 1993;42(2):359-62.
15. Niskanen LK, Tuomi T, Karjalainen J, Groop LC, Uusitupa MIJ. GAD antibodies in NIDDM: ten-year follow-up from the diagnosis. *Diabetes Care* 1995;18(12):1557-1565.

16. Batstra MR, Petersen J, Verwest A, Man de S, Molenaar J, Grobbee D, et al. Can we predict diabetes in schoolchildren? Threshold definition in GAD- and IA2 antibody assays. *Submitted to diabetic medicine* 1998.
17. Dittler J, Seidel D, Schenker M, Ziegler AG. GADIA2-combi Determination as First-Line Screening for Improved Prediction of Type 1 Diabetes in Relatives. *Diabetes* 1998;47(4):592-97.
18. Petersen JS, Hejnaes KR, Moody A, Karlsen AE, Marshall MO, Hoier-Madsen M, et al. Detection of GAD65 antibodies in diabetes and other autoimmune diseases using a simple radioligand assay. *Diabetes* 1994;43(3):459-67.
19. Kim J, Namchuk M, Buchawan T, Fu Q, Jaffe M, Shi Y, et al. Higher autoantibody levels and regognition of a linear NH2-terminal epitope in the autoantigen GAD65, distinguish stiff-man syndrome syndrome from insulin dependent diabetes mellitus. *J Exp Med* 1994;180(2):595-606.
20. Celotti F, Moore GD, Rovescalli AC, Solimena M, Negri-Cesi P, Racagni G. The GABAergic system in the rat oviduct: presence, endocrine regulation and possible localization. *Pharmacol Res* 1989;21(1):103-4.
21. Erdo SL, Joo F, Wolff JR. Immunohistochemical localization of glutamate decarboxylase in the rat oviduct and ovary: further evidence for non-neural GABA systems. *Cell Tissue Res* 1989;255(2):431-4.
22. Hoes AW, Grobbee DE, Valkenburg HA, Lubsen J, Hofman A. Cardiovascular risk and all-cause mortality; a 12 year follow-up study in The Netherlands. *Eur J Epidemiol* 1993;9(3):285-92.
23. Stroink H, Brouwer OF, Arts WF, Geerts AT, Peters AC, van Donselaar CA. The first unprovoked, untreated seizure in childhood: a hospital based study of the accuracy of the diagnosis, rate of recurrence, and long term outcome after recurrence. Dutch study of epilepsy in childhood. *J Neurol Neurosurg Psychiatry* 1998;64(5):595-600.
24. Carpay H, Arts WFM, Geerts H, Brouwer OF, Peters ACB, Donselaar CAv. Epilepsy in Childhood. An audit of clinical practice. 1998;55:668-673.
25. Baekkeskov S, Aanstoot HJ, Christgau S, Reetz A, Solimena M, Cascalho M, et al. Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. *Nature* 1990;347(6289):151-6.
26. Horwitz MS, Bradley LM, Harbertson J, Krahl T, Lee J, Sarvetnick N. Diabetes induced by Coxsackie virus: initiation by bystander damage and not molecular mimicry. *Nat Med* 1998;4(7):781-5.
27. Moran A, Doherty L, Wang X, Thomas W. Abnormal glucose metabolism in cystic fibrosis. *J Pediatr* 1998;133(1):10-17.
28. Marner B, Bille G, Christy M, Damsgaard EM, Garne S, Heinze E, et al. Islet cell cytoplasmic antibodies (ICA) in diabetes and disorders of glucose tolerance. *Diabet Med* 1991;8(9):812-6.

Chapter 5

General Discussion

Type-1 diabetes mellitus is an autoimmune disease that is characterised by a long pre-clinical phase referred to as prediabetes. Prediabetes offers the unique opportunity to prevent the clinical manifestation of type-1 diabetes. This is important since the disease is accompanied by substantial morbidity and may have severe emotional and social consequences for the patients and their families. One third to half of the new cases of type-1 diabetes are diagnosed during childhood [1] and over the last five years diabetes is diagnosed at increasingly younger ages [2-5]. The diabetes control and complications trial (DCCT) has demonstrated that vascular, renal and neurological long-term complications of diabetes can be prevented or delayed by near-normalisation of blood glucose levels. This requires dramatic lifestyle changes and is accompanied by significant weight gain and increased risk on hypoglycaemic episodes [6-10]. It has been repeatedly demonstrated that recurrent hypoglycaemic episodes may result in impaired psychomotor development in young children [11-13]. Thus, especially for young children prevention (or postponement of clinical onset) is better than treatment of type-1 diabetes.

Implementation of preventive measures requires the availability of sensitive and specific screening tools to identify individuals at increased risk to develop the disease. As described in chapter 1.2.3. such tools are available for prediction in first-degree relatives, but their efficacy for prediction in the general population has been less extensively studied. A simple calculation demonstrates that prediction and prevention in first-degree relatives alone does not suffice: Over 90% of new cases of diabetes have no relative with the disease [14]. Applying a preventive measure that has 50% efficacy (as is assumed in the ongoing ENDIT trial) to first-degree relatives at high risk to develop type-1 diabetes (based on their antibody profile) will at maximum reduce the population incidence by 4% (50% (efficacy) of 10% (proportion of familial cases) of 80% (proportion of prediabetic individuals detected by screening [15]) = 4%). At a 13% increase of the diabetes incidence over five years [5], such an effect

on the incidence of type-1 diabetes in the general population is negligible. The work described in this thesis, therefore aims to improve the feasibility of prediction in the general population using ß-cell autoantibodies.

Population screening requires a screening strategy that is:

- Highly reproducible
- Applicable to small serum volumes
- Cheap
- Standardised between laboratories
- Highly specific (low numbers of false positives)
- Highly sensitive (low numbers of false negatives)

There are four types of well-characterised antibodies that can be used for prediction of type-1 diabetes (chapter 1.2.3.). Of these, islet cell antibodies (ICAs) and insulin autoantibodies (IAAs) have been most extensively studied for prediction in first-degree relatives and in the general population [15-24]. The ICA assay has a high predictive sensitivity but is labour intensive, variable between laboratories and requires large serum volumes for quantification. Similar drawbacks account for the detection of IAAs [25]. In addition, the prevalence of IAAs is inversely correlated with age, resulting in variable predictive values in different age groups [21, 26-31]. The recent development of a new micro-assay for IAAs may alter these perspectives [31, 32].

The assays for antibodies against Glutamic Acid Decarboxylase (GAD) and Insulinoma Antigen 2 (IA2), are highly reproducible, require minimal serum volumes for quantification and can be semi-automated [25, 33-35]. Thus, these assays are in essence appropriate for population screening, although additional characterisation and standardisation between laboratories are required. This chapter discusses the results of the practical research on characterisation of antibody screening described in this thesis, gives directions for further research and places the results in an ethical and empirical context.

5.1. Empirical considerations on predictive testing

5.1.1. Epidemiological definitions in predictive testing

An assay is characterised by its sensitivity and specificity. The sensitivity of a test is the frequency of a positive test-result in diseased individuals, while the specificity is defined as the proportion of healthy individuals that test negative. Sensitivity and specificity of a test are inversely proportional and strongly depend on threshold definition. A widely applied technique to define thresholds is Receiver Operated Curve (ROC) analysis (chapter 3.2.) (36). This method is designed to find a mathematical compromise between sensitivity and specificity, irrespective of the aims of screening. Specification of the sensitivity and specificity of a test (validation) enables comparison between laboratories. This is the specific aim of the standardisation workshops that are organised by the immunology of diabetes society (http://www.dem.it/IDSNEWS/antibody_committee.html) (25, 37, 38). The development of WHO standards is an important tool to achieve international validation (39).

For interpretation of the outcome of a test (positive or negative), an additional parameter is required: The predictive value. The positive predictive value of a test is the risk to develop the disease given a positive test result. The negative predictive value is defined as the chance not to get the disease given a negative test result. The predictive value of a test is dependent on its sensitivity and specificity, but strongly depends on the prevalence of the disease in the population as well (Bayes Theorem) (40). The consequences of the Bayes Theorem for extrapolation of diabetes prediction from first-degree relatives to the general population have been described in chapter 3.2. and will be discussed more extensively below.

If a preventive measure, which can prevent diabetes in 50% of the cases treated (as is assumed in the ENDIT study), and a screening strategy that has 54% positive predictive value (15), are implemented in a population of siblings of patients with type-1 diabetes, four siblings will have to be

treated to prevent one case of diabetes. At an antibody frequency of 7% [15], this requires screening for antibodies of 57 siblings for each prevented case. In the general (Finnish!) population (having a 10-fold lower prevalence than siblings), the same screening and prevention approach will have a positive predictive value of 10%. Consequently, 20 children will have to be treated to prevent one case of diabetes, requiring screening of 500 children from the general population. The same principles account when extrapolating data from the Finnish population to the Dutch population (having a threefold lower diabetes incidence), or from studies in the past to the present. Population-based screening is only justified if effective treatment for the disease exists. The 50% success-rate in studies such as ENDIT and the difficulties in the definition of high-risk individuals urge for the development for better screening methods.

5.1.2. Pathophysiological considerations on extrapolation between two populations

Extrapolation of predictive strategies from first-degree relatives to the general population is only legitimate if the underlying disease process in both populations is identical. The results of several epidemiological studies provide circumstantial evidence that this is likely to account for type-1 diabetes:

- The type-1 diabetes risk among first-degree relatives mirrors the geographical pattern of disease incidence [14].
- The risk to contract the disease is similar for relatives of sporadic cases and relatives of familiar cases.
- Although diabetes may become manifest at younger age in those with a positive family history, the clinical manifestation in relatives and sporadic cases is similar [41-45]. The younger presentation of symptoms may be partly due to increased awareness of the symptoms and partly to increased genetic susceptibility.

- In chapter 3.2 we demonstrated that the sensitivity and specificity of screening in the general population are similar to the sensitivity and specificity obtained in family-based populations. Additional data from other studies confirm our observations [21].

Direct evidence on this issue should be derived from comparison of immunological parameters between recent onset familial and sporadic cases, but studies on this topic are virtually lacking. Concerted actions like EURODIAB [46], ICARUS [47] or PARADIGM would form the ideal forum to perform such studies.

5.2. Ethical considerations on predictive testing

It is important to realise that screening brings about risk awareness in healthy individuals, which may be accompanied by significant psychological stress [48, 49]. Therefore the numbers to screen to detect one potential case of diabetes should be kept to a minimum, thus requiring highly sensitive screening strategies. When screening for research purposes no results of screening should be provided to the participants, unless effective preventive measures can be offered.

Another ethical issue encountered on the implementation of screening and intervention in a population is false positive testing and subsequent unjust treatment of individuals. It is obvious that the number of false positive cases should be kept at minimum since each therapy carries the risk of dangerous side effects. The acceptability of unjust treatment is largely dependent on the medical risk and psychological stress introduced by a certain treatment. A good rule to go by is that the social burden and medical risk in the total number of children that has to be screened and treated to prevent one case of diabetes, should not exceed the risk and burden that is introduced by treatment of type-1 diabetes and its complications in one patient.

Another important factor is that, with the currently available screening and intervention tools, only ¼th of the future cases with diabetes can be

prevented (50% screening sensitivity and 50% prevention efficacy). Thus, when designing prediction strategies several ethical considerations should be taken into account and the strategy should be adapted to its specific aims. In this light application of ROC-analysis to establish assay-thresholds reduces an ethical matter to a mathematical compromise representing an oversimplification of the issue.

5.3. Disease progression and antibody formation

Autoantibodies presumably do not play a role in the pathogenesis of disease, but are merely a side effect of the T-cell mediated immune attack [50]. It is not clear in what stage of prediabetes antibodies appear, nor is it known if they are continuously present. The studies on antibodies in newly diagnosed patients and their first-degree relatives described in chapter 2 of this thesis have helped to clarify the complex interrelationship between antibodies and disease progression. The results demonstrate that autoantibodies are not only a valuable prediction tool, but may also be important to decipher the pathogenesis of type-1 diabetes.

Autoantibodies may remain present in the circulation years after clinical manifestation of type-1 diabetes, when presumably (and measurable through c-peptide production) no ß-cells are left (chapter 2) [51]. This observation raises the question whether small-scale ß-cell regeneration may provide a continuous source of ß-cell antigens, thus stimulating antibody formation. However, previous studies have demonstrated that shortly after onset the presence of autoantibodies is correlated to a faster decline of ß-cell function [51-55]. GAD-antibodies may form an exception to this rule, especially in children. In chapter 2 we demonstrated that GAD-antibodies remain present in the circulation longer after onset than IA2 or ICA and that there is no correlation of GAD-antibodies with c-peptide reserve in children. Ludvigson and colleagues and Savola and colleagues have later confirmed our observations [51, 56]. In the same study we

demonstrated that prolonged c-peptide reserve in children who received continuous subcutaneous insulin infusion was not accompanied by increased levels of GAD-antibodies, supporting our hypothesis that the (sustained) presence of GAD-antibodies is not secondary to retained ß-cell function or regeneration. Additionally, we described seroconversions from negative to positive GAD and IA2 antibody levels in patients who had been diagnosed 15 years previously (chapter 2.2.). It is highly unlikely that in these children a sudden revival of the ß-cells occurs. Thus, it is unlikely that the sustained presence of ß-cell antibodies is solely caused by continuous ß-cell regeneration and the observed fluctuations suggest that other (environmental) factors play a role. It would be interesting to study whether the epitopes recognised remain stable through time or differ between two periods of autoimmunity, since this may reveal information on the sources of immunostimulation (see also paragraph 5.6.).

Fluctuations in antibody levels are not unique to patients: in chapter 2.2. we described that first-degree relatives of probands with type-1 diabetes may be transiently positive for GAD- or IA2-antibodies. A striking observation in this study was that conversions from negative to positive or dubious antibody levels most frequently occurred in spring and that, when analysing all first-degree relatives as a whole, antibody levels tended to be higher in spring. Additionally, seroconversions were clustered within families. This clustering in time and space suggests that seroconversions may be caused by environmental factors, possibly viral infections. Hiltunen and colleagues previously described that the appearance of viral antibodies was accompanied by transient positivity for ICAs [57], thus supporting our hypothesis. The interest of this observation lies in the fact that it may indicate that viral infections are involved in initiation of autoimmunity, rather than precipitating the clinical manifestation of diabetes by increased physiological stress. These speculations need to be further verified and we plan to test whether the observed seroconversions are correlated to the presence of enteroviral RNA in serum (in

collaboration with J. Galama en G. Vreugdenhil, University of Nijmegen, Department of Virology).

As described in numerous other studies [15, 28, 58-60], seroconversions for GAD-and IA2 antibodies did not occur simultaneously (neither in relatives nor in patients) in most instances, suggesting that the initiating events are antigen specific. In addition, we described that three cases of diabetes that occurred in one family had a different antibody pattern, but a similar HLA-DR background (chapter 2.2.). This indicates that type-1 diabetes is heterogeneous even within families and that heterogeneity may be determined by the environmental factors encountered. This assumption is also reflected by the discordance in monozygotic twins and high variability in age of onset in concordant twins [61-63]. In addition, Petersen and colleagues described that monozygotic and dizygotic twins do not differ in antibody concordance, suggesting that environmental factors encountered are more important than genetic predisposition [64]. Thus, the primary antigens recognised may be different and the response to ß-cell antigens may be initiated throughout life, suggesting a variable induction and duration of the destructive (prediabetes) process.

In addition, we observed that first-degree relatives might be transiently positive for GAD- or IA2 antibodies. This observation may have serious consequences for diabetes prediction, as described in chapter 5.4.

5.4. Studies on diabetes prediction

5.4.1. Natural course of autoimmunity

The results described in chapter 3.2. of this thesis demonstrate that data on diabetes prediction obtained in family studies can be extrapolated to the general population. However, due to lack of knowledge on the natural course of prediabetes it is not clear when to start screening and how often we should screen.

Antibodies may be transferred transplacentally from the maternal to the fetal circulation, indicating that antibody screening in cord blood samples should be avoided [59, 65]. In the Finnish DIPP study it was demonstrated that, in concordance with the normal clearance rate of IgGs, at 9 months of age maternal antibodies are no longer detectable in the child's circulation [65, 66]. Thus, false positive antibody testing due to maternal antibodies, can be avoided by initiation of screening after 9 months of age. The BABYDIAB, DAISY and DIPP studies aim to evaluate the natural course of ß-cell autoantibodies from birth to the development of type-1 diabetes [28, 58, 65]. These studies have been recently initiated and demonstrate that antibodies may appear before two years of age, in several, but not all, cases followed by the clinical manifestation of diabetes. From these results it is tempting to conclude that single antibody screening early in life may suffice for diabetes prediction. However, the studies lack longer follow-up and Ziegler and colleagues demonstrated that at two years of age 11% of offspring from diabetic parents was positive for 1 or more ß-cell autoantibodies [59]. This exceeds the expected prevalence of diabetes in offspring by three to four times and is higher than the prevalence reported in older offspring, indicating that ß-cell antibodies may disappear later in life. The observations may be explained by the fact that IAAs were included in their study, since it has been described that IAA may be transiently detected in young children [28, 29]. In chapter 2.2. we described that first-degree relatives may be transiently positive for GAD- or IA2 antibodies. Such seroconversions from positive to negative may occur at any age. They possibly represent down-regulation of autoimmunity with subsequent cessation and correction of ß-cell destruction (figure 1.1.), or a transient elevation of antibody levels due to environmental factors (chapter 2.2.). If the latter is the case, these transient positive individuals may cause "false positivity" when implementing a screening strategy based on antibody screening at one single time-point.

In addition, our family study described in chapter 2.2. demonstrate that autoimmunity may be initiated at any age. Other groups have also described conversion to antibody positivity later in life [58, 67-69]. These observations demonstrate the heterogeneity of the disease process, the age of clinical manifestation being determined by the timing of initiation of autoimmunity, the velocity of ß-cell decline and possibly by down-regulation of autoimmunity (figure 1.1.). Thus, a sensitive and specific ß-cell antibody screening strategy should consist of repeated sampling starting early in life (at earliest at 9 months).

5.4.2. Predictive sensitivity and specificity

Apart from the natural course of autoantibodies discussed in the previous paragraph, several other factors may influence sensitivity and specificity of testing.

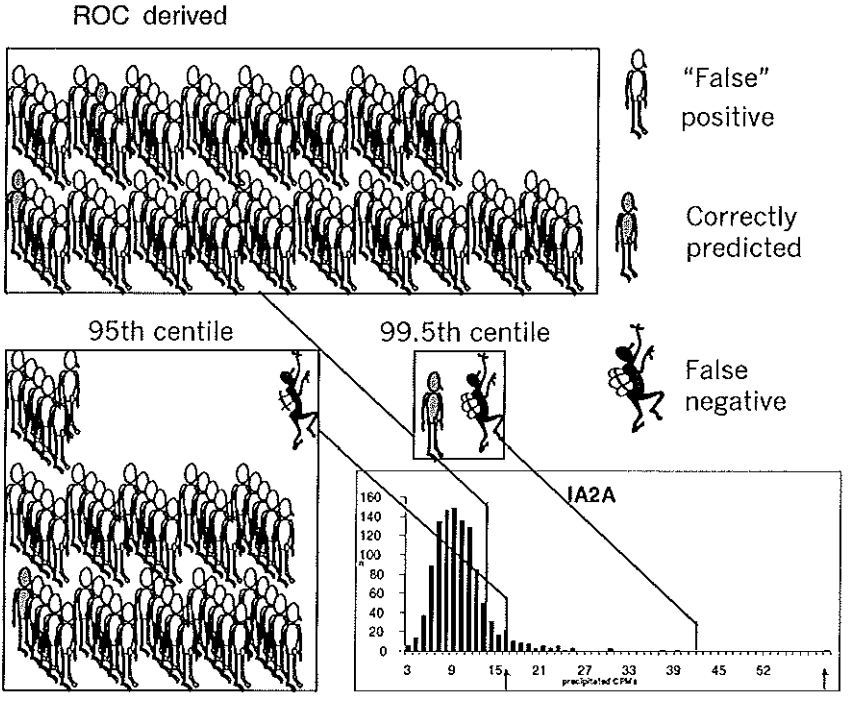
5.4.2.1. Threshold definition

We demonstrated that in our assays the 99.5th centile of a population of schoolchildren resulted in highly specific and sensitive antibody screening, irrespective of age, sex or co-morbidity (chapter 3.2. and 4.1.). In the general population, lowering of thresholds, although established by receiver operated curve analysis, resulted in extreme loss of screening specificity, as demonstrated in figure 5.1.

In chapters 2.2. and 3.3. we demonstrate that a high proportion of patients with long standing diabetes might have GAD-antibody levels between the 95th and 99.5th centile (dubious levels), indicating that autoimmunity is going on or has taken place in the past. The meaning of dubious levels in healthy individuals remains to be established. In our family-study the majority (80%) of the first-degree relatives that had dubious antibody levels at their initial visit turned negative at follow-up visits. This observation implicates that dichotomising of antibody tests is artificial and that dubious antibody levels should be interpreted according

to the indication for antibody testing. Interpretation may require analysis of a follow-up sample.

Figure 5.1. Illustration of the effects of threshold definition on sensitivity and specificity of screening, derived from the results from IA2-antibody screening in the general population (chapter 3.2.).



The numbers of positive individuals at different thresholds are shown. Lowering the threshold from the 99.5th centile to the 95th centile does not improve screening sensitivity, but severely impairs screening specificity (yielding 55 false positives). Using the ROC derived threshold the sensitivity of screening is dramatically improved (from 50-100%), but at the cost of screening sensitivity again.

5.4.2.2. Limited knowledge on target antigens

Several studies have demonstrated that GAD, IA2 and insulin are not the only targets of autoimmunity in patients with type-1 diabetes and individuals in the prediabetic phase [17, 33, 70-72]. Additional targets of the humoral response have been identified but only few are specific for type-1

diabetes and even fewer can be detected in fluid phase assays (paragraph 1.2.3.1.) [73].

GLIMA38 forms an exception to this rule, since it is immunoprecipitated by 19% of sera of newly diagnosed patients with type-1 diabetes, by 14% of patients in the prediabetic phase and not by healthy individuals [74]. GLIMA38 is an amphiphilic membrane glycoprotein of 38 kilodalton, specifically expressed in islet and neuronal cell lines. Although the prevalence of antibodies against GLIMA38 is relatively low, the shared characteristics with both GAD and IA2 (neuroendocrine expression pattern, detectable by immunoprecipitation) suggest that it may be an important target of ß-cell autoimmunity. We are currently working on purification and characterisation of the protein, using phage display techniques (in collaboration with W. van Ewijk and K. Radosevic, Erasmus University and University Hospital Rotterdam, Department of Immunology). Once the molecular identity is known, large-scale screening will be needed to assess the additional value of GLIMA38 antibodies for diabetes prediction. Our current preliminary observations imply that GLIMA38 may be present long before diagnosis of diabetes and disappear rapidly after onset (M.R. Batstra, R. Raatgeep, R. Hagedoorn, G.J. Bruining, H.J. Aanstoot, unpublished observations). The currently applied detection system for GLIMA38 antibodies (immunoprecipitation, followed by SDS-PAGE and autoradiography) is identical to the system previously used to detect GAD- and IA2-antibodies. It is therefore reasonable to assume that, once the protein has been molecularly characterised, detection can be performed in a RBA similar to those used for GAD and IA2 antibodies. This would eventually enable easy semi-automated screening for three autoantibodies at once [34, 35, 75].

In addition to GLIMA38 antibodies, other potential targets of ß-cell autoimmunity are described each year, some of which can be detected in fluid phase assays, others only on western blots [76]. We need to be open minded in evaluating such new antigens. Characterisation of new targets in

ß-cell autoimmunity may not only be important for prediction purposes (improving sensitivity and specificity of screening or providing surrogate endpoints). It may also enable understanding of the pathogenesis of type-1 diabetes, either by identification of early targets of autoimmunity or by dissecting the clinical heterogeneity of the disease.

5.4.2.3. *Non autoimmune mediated type-1 like diabetes*

Besides our inability to detect certain autoantibodies, a small proportion of patients clinically presenting with type-1 diabetes may have suffered ß-cell destruction due to other causes. Studies on several types of diabetes associated with exocrine pancreas diseases such as pancreatitis, cystic fibrosis, haemochromatosis and malnutrition-related diabetes have demonstrated that ß-cell damage may occur without autoantibody formation (chapter 4.1.) [77-84]. In general, these conditions are clearly distinguishable from classical type-1 diabetes and unlikely to be missed, but there may be more diseases with less obvious clinical presentation that are currently diagnosed as type-1 diabetes. In addition, several types of diabetes not associated with ß-cell destruction, such as MODY and mitochondrial diabetes, may present as type-1 diabetes. However, with increasing awareness on these forms of diabetes the likelihood to miss such a differential diagnosis is decreasing and these cases will only account for a negligible amount of false negatives in diabetes prediction studies [85-88]. In our family study two cases within one family developed diabetes without symptoms of autoimmunity (chapter 2.2.). Such cases may represent a non-autoimmune mediated form of insulin dependent diabetes mellitus. It is important to acknowledge such phenomena since they might require a different (preventive) treatment and have a different prognosis. Establishment of a (inter)national diabetes registry may help to further classify diabetes and is necessary to estimate the prevalence of different forms of diabetes.

5.4.2.4. Other markers

Application of other markers than autoantibodies may improve sensitivity and specificity of screening. Genetic testing (in particular HLA-typing) has the highest potency of all alternatives and can be applied for two purposes. The first application is genetic pre-selection of those at increased risk to develop diabetes, thereby identifying approximately 10-15% of the general population, including 60-80% of those who will develop type-1 diabetes (paragraph 1.1.2.3.) [89-91]. Further risk assessment requires repeated measurements of immunological (antibody) markers to improve screening specificity (chapter 3.1.). With the fast developments in molecular biology and the availability of microchips and -arrays for genetic screening, first line screening on genetic risk (HLA susceptibility genes) is easy and relatively cheap and may be implemented in existing neonatal screening programs [90-92]. Hahl and colleagues have demonstrated that such a strategy would save ¾th of the costs of repeated serological screening without genetic pre-selection [91]. However, their calculations were based on ICA screening and screening for GAD- and IA2-antibodies may alter these perspectives. Alternatively, HLA typing could be used to increase screening specificity by excluding those with protective HLA types among those who are positive for any ß-cell antibody [93-95].

Screening for diabetogenic T-cells appears to have the advantage of direct identification of the disease process. However, T-cell assays are labour intensive, require large volumes of fresh blood and are hardly reproducible between laboratories [96]. In addition, auto-reactive T-cells to ß-cell antigens are present in at least 10% of the healthy population [97-101], representing an expensive, unpractical and aspecific disease marker. Thus, T-cells are important for further understanding of type-1 diabetes but are not applicable as predictive or diagnostic marker.

Increasing knowledge on abnormalities in macrophage and dendritic cell maturation in type 1-diabetes may provide an additional predictive and

diagnostic tool [102-105], but further research in human diabetes is required before clinical application.

Metabolic testing is frequently used as a predictive marker for type-1 diabetes. Deteriorating metabolic regulation (measured by a decreased first phase insulin response (FPIR) in an intravenous glucose tolerance test (IVGTT)) indicates that a substantial proportion of the ß-cell mass has already been destroyed and that a person is close to clinical manifestation of the disease [47, 106, 107]. Metabolic testing, therefore, is rather an early diagnostic marker than a predictive marker and its use in diabetes prediction and prevention studies other than as surrogate endpoint is questionable. Alternatively, an increasing pro-insulin/ insulin ratio may be a marker of impending diabetes. However, this measure is inversely correlated to the FPIR and equally represents a late phase of pre-diabetes [108].

The ideal means for diabetes prediction would be direct detection of the inflammatory process in the islets of Langerhans or decreasing islet cell mass (similar to mammography for breast-cancer screening). However, the islets of Langerhans are too small to be imaged in scanning procedures. In one study, using technetium labelled human polyclonal antibodies and gamma camera imaging, there was a significant difference between patients and controls. However, there was a large overlap between normal and diabetic subjects, thus limiting clinical applicability of this technique [109]. Invasive techniques such as endoscopic retrograde pancreatography [110] or pancreas biopsies [111] are ethically and medically unacceptable. In addition, the clinical usefulness of imaging and biopsies other than for research purposes is limited, due to the high costs and ethical burden.

5.5. Other applications of ß-cell antibody screening

5.5.1. Antibodies as a diagnostic tool

Clinical manifestation of type-1 diabetes in childhood is mostly easily discriminated from other types of diabetes due to their mode of inheritance and clinical manifestation [87]. Thus, autoantibodies are generally not necessary for the diagnosis of childhood type-1 diabetes. However, in older patients the clinical manifestation of type-1 diabetes may be less acute with a questionable state of insulin dependency. In these cases discrimination between type-1 and type-2 diabetes is facilitated by the detection of ß-cell antibodies, especially GAD-antibodies [112-118]. Early identification of this form of diabetes, often referred to as Latent Autoimmune Diabetes of Adulthood (LADA) or type-1½ diabetes, and initiation of intensive insulin treatment at clinical diagnosis may help to preserve ß-cell function, thus improving the prognosis [119].

It has been postulated that half of the patients with diabetes aged over fifty are not diagnosed due to the gradual development of the symptoms in these patients [120, 121]. It is not clear how many of these patients actually have type-1 diabetes. The study described in chapter 3.3. aims to invest the prevalence of type-1 diabetes in the Dutch population aged over 50. We demonstrated that the frequency of GAD-antibodies was low (<1%). Among patients with impaired glucose tolerance the antibody frequency was slightly increased and 11% of the insulin treated patients were positive for GAD-antibodies. Thus, we demonstrated that antibody screening in a general population of elderly individuals is not useful and that the frequency of type-1 diabetes among elderly patients presenting with diabetes is lower than previously reported from hospital based studies. This may be explained by selection bias, since patients in whom metabolic regulation is easily maintained by dietary treatment or oral hypoglycaemic drugs will be treated by the general practitioner and will therefore not be included in the hospital based studies. Studies applying

other antibodies are on their way, but in general GAD-antibodies appeared to have the highest prognostic factor in screening for type-1 diabetes in adults presenting with diabetes [113, 122, 123], while IA2-antibodies are associated with classical type-1 diabetes [112].

5.5.2. Antibodies as a prognostic tool

Studies on patients who received a pancreas or islets transplantation have demonstrated that the presence of GAD-antibodies or IA2-antibodies before transplantation or (re)appearance after transplantation is negatively correlated to graft survival [124-127]. Thus, ß-cell antibody testing may be a valuable tool in the evaluation of feasibility of islet or pancreas transplantations in individual patients suffering from type-1 diabetes.

5.6. Technical evaluation of antibody screening

5.6.1. Epitope recognition and application of specific ligands

The vast majority of individuals with newly diagnosed type-1 diabetes and prediabetes has autoantibodies to GAD_{65}, but not GAD_{67}, that recognise conformational epitopes in the middle and c-terminal region of the molecule [128-130]. Sera from patients with stiff man syndrome, a rare neurological disease accompanied by diabetes in approximately 30% of the cases [131], recognise a more extensive array of epitopes, including linear epitopes in the n-terminal region of GAD_{65}. [129-131]. In addition, we have described that, on the T-cell level, certain GAD-epitopes (AA 339-352) may result in a downregulatory immune-response in a patient with stiff man syndrome, but no diabetes [132]. Thus differences in epitope recognition between different diseases may be a tool to improve screening specificity.

IA2-antibodies primarily recognise conformational epitopes in the intracellular domain of IA2 but not IA2-ß [133-137]. Thus, employing assays with limited epitope recognition, due to loss of conformation of the ligand by coating to plastic in ELISA (enzyme linked immunosorbent assay) [138] or exclusive presentation of linear epitopes in Western Blotting, may result in loss of sensitivity for diabetes prediction [130, 139].

In our studies we used two systems for the expression of human recombinant GAD_{65}: in vitro transcription and translation (rabbit TNT reticulocyte lysate system, Promega, Madison, WI, USA) and stable transfection and expression in BHK cells. There appeared to be a discrepancy between recognition of GAD_{65} produced in these systems by a minority of sera, while the DNA template applied was identical [140]. In addition, Aanstoot and colleagues have suggested that GAD_{65} produced in baculovirus is less efficiently recognised by sera from patients with type-1 diabetes (H.J Aanstoot, personal communication) [141]. The latter observation may be predominantly caused by precipitation of GAD_{65} due to the high concentration of GAD_{65} in the preparation and may be partly overcome by addition of carrier proteins. However, the observations warrant further research on (conformational) differences between the GAD-molecules produced in different expression systems. In addition, the magnitude of the observed differences and their meaning for diabetes prediction should be studied, especially since the majority of commercial kits for GAD-antibody detection use GAD_{65} from baculoviral expression systems.

With longer duration of ß-cell autoimmunity epitope spreading occurs [136, 142]. Consequently, early prediabetes may be characterised by less extensive epitope recognition than newly onset diabetes, emphasising that for validation of antibody assays for prediction purposes, prediabetic samples should be included. Kawasaki and colleagues reported that more extensive epitope spreading occurred in relatives who progressed to type-1 diabetes than in those who did not [136]. Thus, limited epitope recognition

may hamper sensitivity of screening to identify early prediabetes, but may on the other hand allow to distinguish between GAD-antibody positive individuals at high and low risk to develop the disease [129, 143]. Practical application of the latter in cross-sectional studies is limited, since antibody recognition differs widely between patients with type-1 diabetes and, although disease-specific epitopes may exist, there is also overlap in the humoral response between diseases [129, 144].

5.6.2. Immunoglobulin isotypes

Using sera from the Finnish DiMe study Petersen and colleagues have demonstrated that the immunoglobulin isotype profile in siblings at low risk reflected a more immature (IgM) and Th2-like (IgE) response compared to siblings at high risk and siblings who converted to diabetes [145]. Additional studies describe a predominant IgG1, IgG3 pattern in progressors and a IgG2, IgG4 pattern in non-progressors to diabetes [146]. Thus specification of antibody isotypes may help to improve screening specificity.

5.6.3. Surrogate endpoints

Alternatively, epitope recognition and antibody isotyping may provide the means to evaluate the effect of preventive measures. This would substantially simplify intervention studies, by limiting the required duration of follow-up. Studies on this issue will accompany both the ENDIT and the DPT intervention studies.

5.7. ß-cell antibody screening now and in the future

Since no preventive measures are at hand, currently predictive screening for type-1 diabetes is of limited use. However, the outcome of the ENDIT study, expected in 2003, and the DPT study, expected in 2005, may

significantly alter these perspectives [147, 148]. In addition, evaluation of new preventive measures requires operative screening strategies. Although there is a strong need for additional knowledge on the timing of initiation of autoimmunity and evolution of the immune response during prediabetes, I would propose a three step screening protocol for the general population:

1) Genetic screening for high (and intermediate) risk HLA-types (with evolving knowledge on interaction between diabetes risk genes, further specified by combined genetic screening). This requires evaluation of the risk conferred by different HLA phenotypes in the target population, a requirement that in The Netherlands can be met by the Kolibrie and ENDIAB studies.
2) Screening those who are at increased genetic risk for the presence of a panel of antibodies that can be detected in a simple, reliable and cheap assay (Currently GAD and IA2 antibodies, but in the future possibly an extended panel of antigens, including GLIMA38).
3) Refining analysis by additional screening for ICA and IAA and specification of the number of antigens recognised. Future technical developments and knowledge on the natural course of autoimmunity may enable analysis of epitope recognition, binding affinity and immunoglobulin isotyping to improve screening specificity.

It is important to recognise that the immune response is a dynamic process that may alter through time, individuals having non-aggressive ß-cell autoimmunity switching to the aggressive type and vice versa. Therefore repeated (each 1 or 2 years?) analysis of step 2 and 3 of the screening protocol may be required in order to achieve a reliable risk assessment. This may provide a way to improve specificity of screening but has the disadvantage that with increasing signs of prediabetes it is likely that the process of ß-cell destruction has become more advanced, possibly resulting in loss of efficacy of intervention (figure 1.2). The need to perform step three will therefore be dependent on the prevention

strategy applied. Alternatively, step 3 may provide a way to monitor the effect of intervention (providing surrogate endpoints).

Experience from diabetes prediction studies learns that it is difficult to motivate individuals from a general population for repeated serum sampling. With the improvement and "micronisation" of the current antibody assay systems capillary sampling has become feasible and in the Dutch healthcare system the first serum samplings could be implemented in vaccination visits (for example at 11 months, 2½ and 3½ years of age). Serum sampling at puberty would involve a separate event and a high drop out rate for these screenings is to be expected. To limit costs and reduce dropout, voluntary participation to screening could be considered. At birth, parents can be given the choice to participate in a screening program, and additional testing of young adults who initially were not included in screening remains possible. By the inclusion of highly motivated individuals, a low dropout percentage in screening is assured, which will lead to a significant decrease of costs.

In contrast to predictive screening, the application of GAD- and IA2-antibodies as diagnostic tool for type-1 diabetes in adults presenting with diabetes is gaining interest. Such screening is useful and may have therapeutical consequences. However, a beneficial effect of early implementation of intensive insulin treatment in those who are positive for GAD-antibodies remains to be established.

5.8. Conclusions

Implementation of preventive measures based on the currently available primary and secondary prediction tools is not feasible. As described in paragraph 5.2., at maximum $\frac{1}{4}^{th}$ of the new cases of diabetes can be prevented by application of the prediction and intervention strategies that are currently under evaluation, at considerably high costs and a considerable number of false positives. New intervention strategies will

therefore first have to be evaluated in newly diagnosed patients to enable dose finding and the definition of surrogate endpoints. However, in these individuals intervention may come too late, resulting in rejection of an intervention strategy that may be effective in prediabetic subjects. Therefore, there is a strong need for the development of highly specific and sensitive prevention strategies. The practical work described in this thesis has contributed to the knowledge on prediction of type-1 diabetes and the clinical course of the disease. The studies have provided data on threshold definition and interpretation of test-results and we have demonstrated that data from family-based studies may be extrapolated to the general population. Further studies on the natural course of autoimmunity are essential to make decisions on the timing and frequency of predictive antibody screening and new markers to estimate progression of autoimmunity need to be developed.

In addition, It has been demonstrated that antibody screening in adults with recent onset diabetes is useful for classification purposes, but that the prevalence of autoimmune diabetes in a population of elderly is low, thus limiting the benefit of antibody screening in such a population.

The studies on the natural course of antibodies in newly diagnosed patients and their first-degree relatives, described in chapter 2 of this thesis, demonstrate that autoantibodies are not only a valuable prediction tool, but may also be important to decipher the pathogenesis of type-1 diabetes. In addition, these studies have emphasised the tremendous heterogeneity of the disease; indicating that preventive measures may have to be as heterogeneous as the disease itself.

References

1. Vandewalle CL, Decraene T, Schuit FC, De Leeuw IH, Pipeleers DG, Gorus FK. Insulin autoantibodies and high titre islet cell antibodies are preferentially associated with the HLA DQA1*0301-DQB1*0302 haplotype at clinical type 1 (insulin-dependent) diabetes mellitus before age 10 years, but not at onset between age 10 and 40 years. The Belgian Diabetes Registry. *Diabetologia* 1993;36(11):1155-62.
2. Karvonen M, Tuomilehto J, Libman I, LaPorte R. A review of the recent epidemiological data on the worldwide incidence of type 1 (insulin-dependent) diabetes mellitus. World Health Organization DIAMOND Project Group. Diabetologia 1993;36(10):883-92.
3. Dahlquist G, Mustonen L. Childhood onset diabetes--time trends and climatological factors. Med J Aust 1995;162(2):111.
4. Tuomilehto J, Virtala E, Karvonen M, Lounamaa R, Pitkaniemi J, Reunanen A, et al. Increase in incidence of insulin-dependent diabetes mellitus among children in Finland. Int J Epidemiol 1995;24(5):984-92.
5. Reeser HM. Epidemiology of childhood diabetes in The Netherlands [PhD]. Leiden State University, 1998.
6. Epidemiology of severe hypoglycemia in the diabetes control and complications trial. The DCCT Research Group [see comments]. Am J Med 1991;90(4):450-9.
7. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. The Diabetes Control and Complications Trial Research Group. N Engl J Med 1993;329(14):977-86.
8. Allaerts W, Tijssen AM, Jeucken PH, Drexhage HA, de Koning J. Influence of folliculo-stellate cells on biphasic luteinizing hormone secretion response to gonadotropin-releasing hormone in rat pituitary cell aggregates. Eur J Endocrinol 1994;130(5):530-9.
9. Dorchy H. What glycemic control can be achieved in young diabetics without residual secretion of endogenous insulin? What is the frequency of severe hypoglycemia and subclinical complications? Arch Pediatr 1994;1(11):970-81.
10. Brink SJ. How to apply the experience from the diabetes control and complications trial to children and adolescents? Ann Med 1997;29(5):425-38.
11. Ryan CM, Williams TM. Effects of insulin-dependent diabetes on learning and memory efficiency in adults. J Clin Exp Neuropsychol 1993;15(5):685-700.
12. Bjorgaas M, Gimse R, Vik T, Sand T. Cognitive function in type 1 diabetic children with and without episodes of severe hypoglycaemia. Acta Paediatr 1997;86(2):148-53.
13. Duvanel CB, Fawer CL, Cotting J, Hohlfeld P, Matthieu JM. Long-term effects of neonatal hypoglycemia on brain growth and psychomotor development in small-for-gestational-age preterm infants. J Pediatr 1999;134(4):492-498.
14. The Eurodiab Ace Study Group and The Eurodiab Ace Substudy 2 Study Group. Familial risk of type I diabetes in European children. Diabetologia 1998;41(10):1151-6.
15. Kulmala P, Savola K, Petersen JS, Vahasalo P, Karjalainen J, Lopponen T, et al. Prediction of insulin-dependent diabetes mellitus in siblings of children with diabetes. A population-based study. The Childhood Diabetes in Finland Study Group. J Clin Invest 1998;101(2):327-36.
16. Bonifacio E, Bingley PJ. Islet autoantibodies and their use in predicting insulin-dependent diabetes. Acta Diabetol 1997;34(3):185-93.
17. Bingley PJ, Bonifacio E, Williams AJ, Genovese S, Bottazzo GF, Gale EA. Prediction of IDDM in the general population: strategies based on combinations of autoantibody markers. Diabetes 1997;46(11):1701-10.

18. Kockum I, Lernmark A, Dahlquist G, Falorni A, Hagopian WA, Landinolsson M, et al. Genetic and immunological findings in patients with newly diagnosed insulin-dependent diabetes mellitus. Horm Metab Res 1996;28(7):344-347.
19. Gorus FK, Goubert P, Semakula C, Vandewalle CL, De Schepper J, Scheen A, et al. IA-2-autoantibodies complement GAD65-autoantibodies in new-onset IDDM patients and help predict impending diabetes in their siblings. The Belgian Diabetes Registry. Diabetologia 1997;40(1):95-9.
20. Aanstoot HJ, Sigurdsson E, Jaffe M, Shi Y, Christgau S, Grobbee D, et al. Value of antibodies to GAD65 combined with islet cell cytoplasmic antibodies for predicting IDDM in a childhood population. Diabetologia 1994;37(9):917-24.
21. Hagopian WA, Sanjeevi CB, Kockum I, Landin-Olsson M, Karlsen AE, Sundkvist G, et al. Glutamate decarboxylase-, insulin-, and islet cell-antibodies and HLA typing to detect diabetes in a general population-based study of Swedish children. J Clin Invest 1995;95(4):1505-11.
22. Verge CF, Gianani R, Kawasaki E, Yu L, Pietropaolo M, Jackson RA, et al. Prediction of type I diabetes in first-degree relatives using a combination of insulin, GAD, and ICA512bdc/IA-2 autoantibodies. Diabetes 1996;45(7):926-33.
23. Knip M, Karjalainen J, Akerblom HK. Islet cell antibodies are less predictive of IDDM among unaffected children in the general population than in sibs of children with diabetes. The Childhood Diabetes in Finland Study Group. Diabetes Care 1998;21(10):1670-3.
24. Schatz D, Krischer J, Horne G, Riley W, Spillar R, Silverstein J, et al. Islet cell antibodies predict insulin-dependent diabetes in United States school age children as powerfully as in unaffected relatives. J Clin Invest 1994;93(6):2403-7.
25. Verge CF, Stenger D, Bonifacio E, Colman PG, Pilcher C, Bingley PJ, et al. Combined use of autoantibodies (IA-2 autoantibody, GAD autoantibody, insulin autoantibody, cytoplasmic islet cell antibodies) in type 1 diabetes: Combinatorial Islet Autoantibody Workshop. Diabetes 1998;47(12):1857-66.
26. Arslanian SA, Becker DJ, Rabin B, Atchison R, Eberhardt M, Cavender D, et al. Correlates of insulin antibodies in newly diagnosed children with insulin-dependent diabetes before insulin therapy. Diabetes 1985;34(9):926-30.
27. Vardi P, Ziegler AG, Mathews JH, Dib S, Keller RJ, Ricker AT, et al. Concentration of insulin autoantibodies at onset of type I diabetes: inverse log-linear correlation with age. Diabetes Care 1988;11:736-739.
28. Roll U, Christie MR, Fuchtenbusch M, Payton MA, Hawkes CJ, Ziegler AG. Perinatal autoimmunity in offspring of diabetic parents: the german multicenter BABY-DIAB study: detection of humoral immune responses to islet antigens in early childhood. Diabetes 1996;45(7):967-973.
29. Ziegler AG, Hillebrand B, Rabl W, Mayrhofer M, Hummel M, Mollenhauer U, et al. On the appearance of islet associated autoimmunity in offspring of diabetic mothers: a prospective study from birth. Diabetologia 1993;36(5):402-8.
30. Vahasalo P, Knip M, Karjalainen J, Tuomilehto-Wolf E, Lounamaa R, Akerblom HK. Islet cell-specific autoantibodies in children with insulin-dependent diabetes mellitus and their siblings at clinical manifestation of the disease. Childhood Diabetes in Finland Study Group. Eur J Endocrinol 1996;135(6):689-95.
31. Williams AJ, Bingley PJ, Bonifacio E, Palmer JP, Gale EA. A novel micro-assay for insulin autoantibodies. J Autoimmun 1997;10(5):473-8.
32. Naserke HE, Dozio N, Ziegler AG, Bonifacio E. Comparison of a novel micro-assay for insulin autoantibodies with the conventional radiobinding assay. Diabetologia 1998;41(6):681-3.
33. Roll U, Ziegler AG. Combined antibody screening for improved prediction of IDDM-- modern strategies. Exp Clin Endocrinol Diabetes 1997;105(1):1-14.
34. Bazzigaluppi E, Bonfanti R, Bingley PJ, Bosi E, Bonifacio E. Capillary Whole Blood Measurement of Islet Autoantibodies. Diabetes Care 1999;22(2):275-279.

35. Dittler J, Seidel D, Schenker M, Ziegler AG. GADIA2-combi Determination as First-Line Screening for Improved Prediction of Type 1 Diabetes in Relatives. Diabetes 1998;47(4):592-97.
36. Zweig MH, Campbell G. Receiver-operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine. Clin Chem 1993;39(4):561-77.
37. Schmidli RS, Colman PG, Bonifacio E, Bottazzo GF, Harrison LC. High level of concordance between assays for glutamic acid decarboxylase antibodies. The First International Glutamic Acid Decarboxylase Antibody Workshop. Diabetes 1994;43(8):1005-9.
38. Schmidli RS, Colman PG, Bonifacio E. Disease sensitivity and specificity of 52 assays for glutamic acid decarboxylase antibodies. The Second International GADAB Workshop. Diabetes 1995;44(6):636-40.
39. Lernmark A, Kolb H, Mire-Sluis T. Towards a World Health Organization (WHO) approved standard sample for islet cell antibodies, GAD65 and IA-2 autoantibodies. Diabetologia 1999;42(3):381-2.
40. Sturmans F. Epidemiology, methods and applications (Dutch). Nijmegen: Dekker & van de Vegt, 1986.
41. Dahlquist GG, Mustonen LR. Clinical onset characteristics of familial versus nonfamilial cases in a large population-based cohort of childhood-onset diabetes patients. Diabetes Care 1995;18(6):852-4.
42. Lorenzen T, Pociot F, Hougaard P, Nerup J. Long-term risk of IDDM in first-degree relatives of patients with IDDM. Diabetologia 1994;37(3):321-7.
43. Pociot F, Norgaard K, Hobolth N, Andersen O, Nerup J. A nationwide population-based study of the familial aggregation of type 1 (insulin-dependent) diabetes mellitus in Denmark. Danish Study Group of Diabetes in Childhood. Diabetologia 1993;36(9):870-5.
44. Metcalfe MA, Baum JD. Family characteristics and insulin dependent diabetes. Arch Dis Child 1992;67(6):731-6.
45. O'Leary LA, Dorman JS, LaPorte RE, Orchard TJ, Becker DJ, Kuller LH, et al. Familial and sporadic insulin-dependent diabetes: evidence for heterogeneous etiologies? Diabetes Res Clin Pract 1991;14(3):183-90.
46. Green A, Gale EA, Patterson CC. Incidence of childhood onset insulin dependent diabetes mellitus:the EURODIAB ACE Study. Lancet 1992;339(8798):905-909.
47. Bingley PJ. Interactions of age, islet cell antibodies, insulin autoantibodies, and first-phase insulin response in predicting risk of progression to IDDM in ICA+ relatives: the ICARUS data set. Islet Cell Antibody Register Users Study. Diabetes 1996;45(12):1720-8.
48. Wagner A, A T, Bruining G, Aanstoot H, Tiems I, Blondeau M, et al. Preliminary experience with predictive testing for insulin-dependent diabetes mellitus. Lancet 1995;346:380-381.
49. Weber B, Roth R. Psychological aspects in diabetes prevention trials. Ann Med 1997;29(5):461-7.
50. Atkinson MA, Maclaren NK. The pathogenesis of insulin-dependent diabetes mellitus. N Engl J Med 1994;331(21):1428-36.
51. Savola K, Sabbah E, Kulmala P, Vahasalo P, Ilonen J, Knip M. Autoantibodies associated with Type I diabetes mellitus persist after diagnosis in children. Diabetologia 1998;41(11):1293-7.
52. Komulainen J, Knip M, Lounamaa R, Vahasalo P, Karjalainen J, Sabbah E, et al. Poor beta-cell function after the clinical manifestation of type 1 diabetes in children initially positive for islet cell specific autoantibodies. The Childhood Diabetes in Finland Study Group. Diabet Med 1997;14(7):532-7.
53. Salardi S, Cacciari E, Steri L, Donati S, Mandini M, Gualandi S, et al. An 8-year follow-up of anti-insulin antibodies in diabetic children: relation to insulin autoantibodies, HLA type, beta-cell function, clinical course and type of insulin therapy. Acta Paediatrica 1995;84(6):639-645.

54. Urakami T, Miyamoto Y, Matsunaga H, Owada M, Kitagawa T. Serial changes in the prevalence of islet cell antibodies and islet cell antibody titer in children with IDDM of abrubt or slow onset. Diabetes Care 1995;18(8):1095-1099.
55. Wallensteen M, Dahlquist G, Persson B, Landin-Olsson M, Lernmark A, Sundkvist G, et al. Factors influencing the magnitude, duration, and rate of fall of B-cell function in type 1 (insulin-dependent) diabetic children followed for two years from their clinical diagnosis. Diabetologia 1988;31(9):664-9.
56. Ludvigsson J, Hellstrom S. Autoantibodies in relation to residual insulin secretion in children with IDDM. Diabetes Res Clin Pract 1997;35(2-3):81-9.
57. Hiltunen M, Hyoty H, Knip M, Ilonen J, Reijonen H, Vahasalo P, et al. Islet cell antibody seroconversion in children is temporally associated with enterovirus infections. Childhood Diabetes in Finland (DiMe) Study Group. J Infect Dis 1997;175(3):554-60.
58. Yu L, Rewers M, Gianani R, Kawasaki E, Zhang Y, Verge C, et al. Antiislet autoantibodies usually develop sequentially rather than simultaneously. J Clin Endocrinol Metab 1996;81(12):4264-7.
59. Ziegler AG, Hummel M, Schenker M, Bonifacio E. Autoantibody appearance and risk for development of childhood diabetes in offspring of parents with type 1 diabetes: the 2-year analysis of the German BABYDIAB Study. Diabetes 1999;48(3):460-8.
60. Rewers M, Norris JM, Eisenbarth GS, Erlich HA, Beaty B, Klingensmith G, et al. Beta-cell autoantibodies in infants and toddlers without IDDM relatives: diabetes autoimmunity study in the young (DAISY). J Autoimmun 1996;9(3):405-10.
61. Verge CF, Gianani R, Yu L, Pietropaolo M, Smith T, Jackson RA, et al. Late progression to diabetes and evidence for chronic beta-cell autoimmunity in identical twins of patients with type I diabetes. Diabetes 1995;44(10):1176-9.
62. Kumar D, Gemayel NS, Deapen D, Kapadia D, Yamashita PH, Lee M, et al. North-American twins with IDDM. Genetic, etiological, and clinical significance of disease concordance according to age, zygosity, and the interval after diagnosis in first twin. Diabetes 1993;42(9):1351-63.
63. Kaprio J, Tuomilehto J, Koskenvuo M, Romanov K, Reunanen A, Eriksson J, et al. Concordance for type 1 (insulin-dependent) and type 2 (non-insulin-dependent) diabetes mellitus in a population-based cohort of twins in Finland. Diabetologia 1992;35(11):1060-7.
64. Petersen JS, Kyvik KO, Bingley PJ, Gale EA, Green A, Dyrberg T, et al. Population based study of prevalence of islet cell autoantibodies in monozygotic and dizygotic Danish twin pairs with insulin dependent diabetes. Brit Med J 1997;314(7094):1575-9.
65. Hamalainen A, Ronkainen M, Akerblom H, Knip M. Elimination of transplacentally transferred antibodies associated with type-1 diabetes in siblings of affected families. 25th ISPAD meeting, Noordwijkerhout, The Netherlands. Diab Res Clin Practice 1999;44(S1):015.
66. Martikainen A, Saukkonen T, Kulmala PK, Reijonen H, Ilonen J, Teramo K, et al. Disease-associated antibodies in offspring of mothers with IDDM. Diabetes 1996;45(12):1706-10.
67. Bonifacio E, Bingley PJ, Shattock M, Dean BM, Dunger D, Gale EA, et al. Quantification of islet-cell antibodies and prediction of insulin- dependent diabetes. Lancet 1990;335(8682):147-9.
68. Roll U, Christie MR, Standl E, Ziegler AG. Associations of anti-GAD antibodies with islet cell antibodies and insulin autoantibodies in first-degree relatives of type I diabetic patients. Diabetes 1994;43(1):154-60.
69. Riley WJ, Maclaren NK, Krischer J, Spillar RP, Silverstein JH, Schatz DA, et al. A prospective study of the development of diabetes in relatives of patients with insulin-dependent diabetes. N Engl J Med 1990;323(17):1167-72.
70. Richter W, Seissler J, Northemann W, Wolfahrt S, Meinck HM, Scherbaum WA. Cytoplasmic islet cell antibodies recognize distinct islet antigens in IDDM but not in stiff man syndrome. Diabetes 1993;42(11):1642-8.

71. Bonifacio E, Genovese S, Braghi S, Bazzigaluppi E, Lampasona V, Bingley PJ, et al. Islet autoantibody markers in IDDM: risk assessment strategies yielding high sensitivity. Diabetologia 1995;38(7):816-22.
72. Verge CF, Gianani R, Kawasaki E, Yu L, Pietropaolo M, Chase HP, et al. Number of autoantibodies (against insulin, GAD or ICA512/IA2) rather than particular autoantibody specificities determines risk of type I diabetes. J Autoimmun 1996;9(3):379-83.
73. Lampasona V, Ferrari M, Bosi E, Pastore MR, Bingley PJ, Bonifacio E. Sera from patients with IDDM and healthy individuals have antibodies to ICA69 on western blots but do not immunoprecipitate liquid phase antigen. J Autoimmun 1994;7(5):665-74.
74. Aanstoot HJ, Kang SM, Kim J, Lindsay LA, Roll U, Knip M, et al. Identification and characterization of glima 38, a glycosylated islet cell membrane antigen, which together with GAD65 and IA2 marks the early phases of autoimmune response in type 1 diabetes. J Clin Invest 1996;97(12):2772-83.
75. Wiest-Ladenburger U, Hartmann R, Hartmann U, Berling K, Bohm BO, Richter W. Combined analysis and single-step detection of GAD65 and IA2 autoantibodies in IDDM can replace the histochemical islet cell antibody test. Diabetes 1997;46(4):565-71.
76. Bonifacio E, Christie MR. Islet cell antigens in the prediction and prevention of insulin- dependent diabetes mellitus. Ann Med 1997;29(5):405-12.
77. Conte D, Manachino D, Colli A, Guala A, Aimo G, Andreoletti M, et al. Prevalence of genetic hemochromatosis in a cohort of Italian patients with diabetes mellitus. Ann Intern Med 1998;128(5):370-3.
78. Cucinotta D, Arrigo T, De Luca F, Di Benedetto A, Lombardo F, Scoglio R, et al. Metabolic and clinical events preceding diabetes mellitus onset in cystic fibrosis. Eur J Endocrinol 1996;134(6):731-6.
79. Dabadghao P, Bhatia E, Bhatia V, Jayaraj K, Colman PG. Islet-cell antibodies in malnutrition-related diabetes mellitus from north India. Diabetes Res Clin Pract 1996;34(2):73-8.
80. Lanng S, Thorsteinsson B, Pociot F, Marshall MO, Madsen HO, Schwartz M, et al. Diabetes mellitus in cystic fibrosis: genetic and immunological markers. Acta Paediatr 1993;82(2):150-4.
81. Larsen S. Diabetes mellitus secondary to chronic pancreatitis. Dan Med Bull 1993;40(2):153-62.
82. Marner B, Bille G, Christy M, Damsgaard EM, Garne S, Heinze E, et al. Islet cell cytoplasmic antibodies (ICA) in diabetes and disorders of glucose tolerance. Diabet Med 1991;8(9):812-6.
83. Mohan V, Deepa R, Bhatia E, Singh AK, Hitman GA, Zimmet PZ, et al. Antibodies to pancreatic islet cell antigens in diabetes seen in Southern India with particular reference to fibrocalculous pancreatic diabetes. Diabet Med 1998;15(2):156-9.
84. Stutchfield PR, O'Halloran SM, Smith CS, Woodrow JC, Bottazzo GF, Heaf D. HLA type, islet cell antibodies, and glucose intolerance in cystic fibrosis. Arch Dis Child 1988;63(10):1234-9.
85. Velho G, Byrne MM, Clement K, Sturis J, Pueyo ME, Blanche H, et al. Clinical phenotypes, insulin secretion, and insulin sensitivity in kindreds with maternally inherited diabetes and deafness due to mitochondrial tRNALeu(UUR) gene mutation. Diabetes 1996;45(4):478-87.
86. Maassen JA, Jansen JJ, Kadowaki T, van den Ouweland JM, t Hart LM, Lemkes HH. The molecular basis and clinical characteristics of Maternally Inherited Diabetes and Deafness (MIDD), a recently recognized diabetic subtype. Exp Clin Endocrinol Diabetes 1996;104(3):205-11.
87. Dussoix P, Vaxillaire M, Iynedjian PB, Tiercy JM, Ruiz J, Spinas GA, et al. Diagnostic heterogeneity of diabetes in lean young adults: classification based on immunological and genetic parameters. Diabetes 1997;46(4):622-31.

88. Ledermann HM. Maturity-onset diabetes of the young (MODY) at least ten times more common in europe than previously assumed? Diabetologia 1995;38(12):1482.
89. Ronningen KS. Genetics in the prediction of insulin-dependent diabetes mellitus: from theory to practice. Ann Med 1997;29(5):387-92.
90. Simell T, Hahl J, Muona P, Simell O. Direct costs of genetic prediction of IDDM and subsequent ICA follow-up of the at-risk children vs. direct costs of repeated unselected ICA screenings. Autoimmunity 1996;24(S1):A190.
91. Hahl J, Simell T, Ilonen J, Knip M, Simell O. Costs of predicting IDDM. Diabetologia 1998;41(1):79-85.
92. Ilonen J, Reijonen H, Herva E, Sjoroos M, Iitia A, Lovgren T, et al. Rapid HLA-DQB1 genotyping for four alleles in the assessment of risk for IDDM in the Finnish population. The Childhood Diabetes in Finland (DiMe) Study Group. Diabetes Care 1996;19(8):795-800.
93. Kockum I, Sanjeevi CB, Eastman S, Landinolsson M, Dahlquist G, Lernmark A, et al. Population analysis of protection by HLA-DR and DQ genes from insulin-dependent diabetes mellitus in swedish children with insulin-dependent diabetes and controls. Eur J Immunogenet 1995;22(6):443-465.
94. Morel PA, Dorman JS, Todd JA, McDevitt HO, Trucco M. Aspartic acid at position 57 of the HLA-DQ beta chain protects against type I diabetes: a family study. Proc Natl Acad Sci U S A 1988;85(21):8111-5.
95. Pugliese A, Gianani R, Moromisato R, Awdeh ZL, Alper CA, Erlich HA, et al. HLA-DQB1*0602 is associated wit dominant protection from diabetes even among islet cell antibody positive first degree relatives of patients with IDDM. Diabetes 1995;44:608-613.
96. Roep BO. Standardization of T-cell assays in Type I diabetes. Immunology of Diabetes Society T-cell Committee. Diabetologia 1999;42(5):636-7.
97. Harrison LC, De Aizpurua H, Loudovaris T, Campbell IL, Cebon JS, Tait BD, et al. Reactivity to human islets and fetal pig proislets by peripheral blood mononuclear cells from subjects with preclinical and clinical insulin- dependent diabetes. Diabetes 1991;40(9):1128-33.
98. Roep BO, Kallan AA, Duinkerken G, Arden SD, Hutton JC, Bruining GJ, et al. T-cell reactivity to beta-cell membrane antigens associated with beta- cell destruction in IDDM. Diabetes 1995;44(3):278-83.
99. Atkinson MA, Kaufman DL, Campbell L, Gibbs KA, Shah SC, Bu DF, et al. Response of peripheral-blood mononuclear cells to glutamate decarboxylase in insulin-dependent diabetes. Lancet 1992;339(8791):458-9.
100. Bonifacio E, Boitard C. T-cell reactivity to 38 kD insulin-secretory granule protein in patients with recent onset type 1 diabetes. J Endocrinol Invest 1994;17(7):559-63.
101. Roep BO. T-cell responses to autoantigens in IDDM. The search for the Holy Grail. Diabetes 1996;45(9):1147-56.
102. Simons PJ, Delemarre FG, Drexhage HA. Antigen-presenting dendritic cells as regulators of the growth of thyrocytes: a role of interleukin-1beta and interleukin-6. Endocrinology 1998;139(7):3148-56.
103. Delemarre FG, Simons PJ, de Heer HJ, Drexhage HA. Signs of immaturity of splenic dendritic cells from the autoimmune prone biobreeding rat: consequences for the in vitro expansion of regulator and effector T cells. J Immunol 1999;162(3):1795-801.
104. Jansen A, van Hagen M, Drexhage HA. Defective maturation and function of antigen-presenting cells in type 1 diabetes. Lancet 1995;345(8948):491-2.
105. Jansen A, Homo-Delarche F, Hooijkaas H, Leenen PJ, Dardenne M, Drexhage HA. Immunohistochemical characterization of monocytes-macrophages and dendritic cells involved in the initiation of the insulitis and beta- cell destruction in NOD mice. Diabetes 1994;43(5):667-75.
106. Knip M, Vahasalo P, Karjalainen J, Lounamaa R, Akerblom HK. Natural history of preclinical IDDM in high risk siblings. Childhood Diabetes in Finland Study Group. Diabetologia 1994;37(4):388-93.

107. Colman PG, McNair P, Margetts H, Schmidli RS, Werther GA, Alford FP, et al. The Melbourne Pre-Diabetes Study: prediction of type 1 diabetes mellitus using antibody and metabolic testing. Med J Aust 1998;169(2):81-4.
108. Roder ME, Knip M, Hartling SG, Karjalainen J, Akerblom HK, Binder C. Disproportionately elevated proinsulin levels precede the onset of insulin-dependent diabetes mellitus in siblings with low first phase insulin responses. The Childhood Diabetes in Finland Study Group. J Clin Endocrinol Metab 1994;79(6):1570-5.
109. Barone R, Procaccini E, Chianelli M, Annovazzi A, Fiore V, Hawa M, et al. Prognostic relevance of pancreatic uptake of technetium-99m labelled human polyclonal immunoglobulins in patients with type 1 diabetes. Eur J Nucl Med 1998;25(5):503-8.
110. Nakanishi K, Kobayashi T, Miyashita H, Okubo M, Sugimoto T, Murase T, et al. Exocrine pancreatic ductograms in insulin-dependent diabetes mellitus. Am J Gastroenterol 1994;89(5):762-6.
111. Hanafusa T, Miyazaki A, Miyagawa J, Tamura S, Inada M, Yamada K, et al. Examination of islets in the pancreas biopsy specimens from newly diagnosed type 1 (insulin-dependent) diabetic patients. Diabetologia 1990;33(2):105-11.
112. Seissler J, de Sonnaville JJ, Morgenthaler NG, Steinbrenner H, Glawe D, Khoo-Morgenthaler UY, et al. Immunological heterogeneity in type I diabetes: presence of distinct autoantibody patterns in patients with acute onset and slowly progressive disease. Diabetologia 1998;41(8):891-7.
113. Hagopian WA, Karlsen AE, Gottsater A, Landin-Olsson M, Grubin CE, Sundkvist G, et al. Quantitative assay using recombinant human islet glutamic acid decarboxylase (GAD65) shows that 64K autoantibody positivity at onset predicts diabetes type. J Clin Invest 1993;91(1):368-74.
114. Zimmet P. Antibodies to glutamic acid decarboxylase in the prediction of insulin dependency. Diabetes Res Clin Pract 1996;34 Suppl:S125-31.
115. Tuomi T, Groop LC, Zimmet PZ, Rowley MJ, Knowles W, Mackay IR. Antibodies to glutamic acid decarboxylase reveal latent autoimmune diabetes mellitus in adults with a non-insulin-dependent onset of disease. Diabetes 1993;42(2):359-62.
116. Turner R, Stratton I, Horton V, Manley S, Zimmet P, Mackay IR, et al. UKPDS 25: autoantibodies to islet-cell cytoplasm and glutamic acid decarboxylase for prediction of insulin requirement in type 2 diabetes. UK Prospective Diabetes Study Group. Lancet 1997;350(9087):1288-93.
117. Vandewalle CL, Falorni A, Svanholm S, Lernmark A, Pipeleers DG, Gorus FK. High diagnostic sensitivity of glutamate decarboxylase autoantibodies in insulin-dependent diabetes mellitus with clinical onset between age 20 and 40 years. The Belgian Diabetes Registry. J Clin Endocrinol Metab 1995;80(3):846-51.
118. Bruno G, Salvia AD, Arcari R, Borra M, Grosso N, Carta Q, et al. Clinical, immunological, and genetic heterogeneity of diabetes in an Italian population-based cohort of lean newly diagnosed patients aged 30-54 years. Piedmont Study Group for Diabetes Epidemiology. Diabetes Care 1999;22(1):50-5.
119. Linn T, Ortac K, Laube H, Federlin K. Intensive therapy in adult insulin-dependent diabetes mellitus is associated with improved insulin sensitivity and reserve: a randomized, controlled, prospective study over 5 years in newly diagnosed patients. Metabolism 1996;45(12):1508-13.
120. Cowie CC, Harris MI, Eberhardt MS. Frequency and determinants of screening for diabetes in the U.S. Diabetes Care 1994;17(10):1158-63.
121. Harris MI, Klein R, Welborn TA, Knuiman MW. Onset of NIDDM occurs at least 4-7 years before clinical diagnosis. Diabetes Care 1992;15:815-819.
122. Zimmet PZ, Elliott RB, Mackay IR, Tuomi T, Rowley MJ, Pilcher CC, et al. Autoantibodies to glutamic acid decarboxylase and insulin in islet cell antibody positive presymptomatic type 1 diabetes mellitus: frequency and segregation by age and gender. Diabet Med 1994;11(9):866-71.

123. Niskanen LK, Tuomi T, Karjalainen J, Groop LC, Uusitupa MIJ. GAD antibodies in NIDDM: ten-year follow-up from the diagnosis. Diabetes Care 1995;18(12):1557-1565.
124. Bosi E, Bottazzo GF, Secchi A, Pozza G, Shattock M, Saunders A, et al. Islet cell autoimmunity in type I diabetic patients after HLA-mismatched pancreas transplantation. Diabetes 1989;1:82-4.
125. Esmatjes E, Rodriguez-Villar C, Ricart MJ, Casamitjana R, Martorell J, Sabater L, et al. Recurrence of immunological markers for type 1 (insulin-dependent) diabetes mellitus in immunosuppressed patients after pancreas transplantation. Transplantation 1998;66(1):128-31.
126. Jaeger C, Brendel MD, Hering BJ, Eckhard M, Federlin K, Bretzel RG. IA-2 antibodies are only positive in association with GAD 65 and islet cell antibodies in islet transplanted insulin-dependent diabetes mellitus patients. Transplant Proc 1998;30(2):659-60.
127. Jaeger C, Hering BJ, Hatziagelaki E, Federlin K, Bretzel RG. Glutamic acid decarboxylase antibodies are more frequent than islet cell antibodies in islet transplanted IDDM patients and persist or occur despite immunosuppression. J Mol Med 1999;77(1):45-8.
128. Ujihara N, Daw K, Gianani R, Boel E, Yu L, Powers AC. Identification of glutamic acid decarboxylase autoantibody heterogeneity and epitope regions in type I diabetes. Diabetes 1994;43(8):968-75.
129. Daw K, Powers A. Two distinct glutamic acid decarboxylase auto-antibody specificities in IDDM target different epitopes. Diabetes 1995;44(2):216-220.
130. Kim J, Namchuk M, Bugawan T, Fu Q, Jaffe M, Shi Y, et al. Higher autoantibody levels and recognition of a linear NH2-terminal epitope in the autoantigen GAD65, distinguish stiff-man syndrome from insulin-dependent diabetes mellitus. J Exp Med 1994;180(2):595-606.
131. Aanstoot H, Michaels A, Christgau S, Shi Y, Kim J, Baekkeskov S. Stiff-Man Syndrome and Insulin Dependent Diabetes Mellitus: Similarities and Differences in Autoimmune Reactions. In: Layzer RB, editor. Motor Unit Hyperactivity States. New York: Raven Press, 1993:53-67.
132. Schloot NC, Batstra MC, Duinkerken G, De Vries RR, Dyrberg T, Chaudhuri A, et al. GAD65-Reactive T cells in a non-diabetic stiff-man syndrome patient. J Autoimmun 1999;12(4):289-96.
133. Naserke HE, Ziegler AG, Lampasona V, Bonifacio E. Early development and spreading of autoantibodies to epitopes of IA-2 and their association with progression to type 1 diabetes. J Immunol 1998;161(12):6963-9.
134. Lampasona V, Bonifacio E, Bingley P, Gale E. Development of IA-2 autoantibody epitope specifities in sequential samples from relatives. autoimmunity 1996;24, S1:A028.
135. Notkins AL, Zhang B, Matsumoto Y, Lan MS. Comparison of IA-2 with IA-2beta and with six other members of the protein tyrosine phosphatase family: recognition of antigenic determinants by IDDM sera. J Autoimmun 1997;10(3):245-50.
136. Kawasaki E, Yu L, Rewers MJ, Hutton JC, Eisenbarth GS. Definition of multiple ICA512/phogrin autoantibody epitopes and detection of intramolecular epitope spreading in relatives of patients with type 1 diabetes. Diabetes 1998;47(5):733-42.
137. Zhang B, Lan MS, Notkins AL. Autoantibodies to IA-2 in IDDM: location of major antigenic determinants. Diabetes 1997;46(1):40-3.
138. Papouchado ML, Valdez SN, Ermacora MR, Ganan S, Poskus E. Highly-sensitive and specific enzyme-linked immunosorbent assays for GAD65 autoantibodies using a thioredoxin-GAD65 fusion antigen. J Immunol Methods 1997;207(2):169-78.
139. Tuomi T, Rowley MJ, Knowles WJ, Chen QY, McAnally T, Zimmet PZ, et al. Autoantigenic properties of native and denatured glutamic acid decarboxylase: evidence for a conformational epitope. Clin Immunol Immunopathol 1994;71(1):53-9.

140. Batstra M, van Driel A, Verwest A, Bruining G, Dyrberg T, Aanstoot H. Is there a discrepancy between the widely applied RIA and convetional immunoprecipitation /SDS-PAGE assay for GAD-antibodies? 2nd IDS meeting, Canberra, Australia. Autoimmunity 1996;24(S1):A1151.
141. Aanstoot H. Identification,chracterization and application of autoantigens in type I diabetes mellitus [PhD thesis]. Erasmus University Rotterdam, 1993.
142. Richter W, Jury K, Loeffler D, Manfras B, Eiermann T, Boehm B. Immunoglobulin variable gene analysis of human autoantibodies reveals antigen-driven immune response to glutamate decarboxylase in type 1 diabetes mellitus. Eur J Immunol. 1995;25(6):1703-12.
143. Bjork E, Velloso LA, Kampe O, Karlsson FA. GAD autoantibodies in IDDM, stiff-man syndrome, and autoimmune polyendocrine syndrome type I recognize different epitopes. Diabetes 1994;43(1):161-5.
144. Davenport C, Radford PM, Al-Bukhari TA, Lai M, Bottazzo GF, Todd I. Heterogeneity in the occurrence of a subset of autoantibodies to glutamic acid decarboxylase in autoimmune polyendocrine patients with islet cell antibodies. Clin Exp Immunol 1998;111(3):497-505.
145. Petersen JS, Kulmala P, Clausen JT, Knip M, Dyrberg T. Progression to type 1 diabetes is associated with a change in the immunoglobulin isotype profile of autoantibodies to glutamic acid decarboxylase (GAD65). Childhood Diabetes in Finland Study Group. Clin Immunol 1999;90(2):276-81.
146. Couper JJ, Harrison LC, Aldis JJ, Colman PG, Honeyman MC, Ferrante A. IgG subclass antibodies to glutamic acid decarboxylase and risk for progression to clinical insulin-dependent diabetes. Hum Immunol 1998;59(8):493-9.
147. Gale EA. Theory and practice of nicotinamide trials in pre-type 1 diabetes. J Pediatr Endocrinol Metab 1996;9(3):375-9.
148. Gerstein HC, Simpson JR, Atkinson S, Taylor DW, VanderMeulen J. The Diabetes Prevention Trial-Type 1 diabetes (DPT-1): implementation of screening and staging of relatives. DPT-1 Study Group. Feasibility and acceptability of a proposed infant feeding intervention trial for the prevention of type I diabetes. Transplant Proc 1995;27(6):3377.

Chapter 6

List of abbreviations

Summary

Samenvatting

6.1. List of abbreviations

ADA : American Diabetes Association
APC : Antigen Presenting Cell
BDA : British Diabetes Association
BHK cells : Baby Hamster Kidney cells
BMI : Body Mass Index
BSA : Bovine Serum Albumin
ccpep : C-peptide secretion
CI : Confidence Interval
cIMP : conventional Immunoprecipitation evaluated by SDS-PAGE and fluorography
CMV : Cytomegalovirus
CPM : Counts Per Minute (precipitated activity in RBA)
CSII : Continuous Subcutaneous Insulin Infusion
CTLA4 : Cytotoxic Lymphocyte Associated Protein – 4
DCCT : Diabetes Control and Complications Trial
DPT : Diabetes Prevention Trial
ELISA : Enzyme Linked Immuno Sorbent Assay
ENDIT : European Nicotinamide Diabetes Intervention Trial
FPIR : First Phase Insulin Response
GAD : Glutamic Acid Decaboxylase
GAD_{65} : The 65 kD isoform of Glutamic acid decaboxylase
GAD65-A : GAD-antibodies
GAD_{67} : The 67 kD isoform of Glutamic acid decaboxylase
GAD-Ab : GAD-antibodies
GAD-index : Relative units of GAD-antibody levels
GDM : Gestational Diabetes Mellitus
GLIMA38 : Glycosylated Islet Membrane Antigen with a molecular mass of 38 kilodalton
HLA : Human Leucocyte Antigen
IA2 : Insulinoma antigen 2
IA2-index : Relative units of IA2-antibody levels
IA2-ß : Phogrin
IAA : Insulin Auto-Antibodies
ICA : Islet Cell Antibodies
ICA69 : Islet Cell Antigen 69
ICAM : Intracellular cell adhesion molecule
IDDM : Insulin Dependent Diabetes Mellitus
IDDM1-15 : Gene loci involved in type-1 diabetes
IFN : Interferon
IgA : Immunoglobulin A
IgE : Immunoglobulin E
IgG : Immunoglobulin G
IgM : Immunoglobulin M
IGT : Impaired Glucose Tolerance

IL	: Interleukin
INS VNTR	: Variable Number of Tandem Repeats in the promotor region of the insulin gene
IVGTT	: Intravenous Glucose Tolerance Test
JDF	: Juvenile Diabetes Foundation
JDF-units	: Standardization units for ICA
LADA	: Latent Autoimmune Diabetes of Adults
lnGAD	: Log transformed GAD-index
lnIA2	: Log transformed IA2-index
MCTD	: Mixed Connective Tissue Disease
MHC	: Major Hisocompatibility Complex
MODY	: Maturity Onset Diabetes of the Young
NIDDM	: Non Insulin Dependent Diabetes Mellitus
NOD-mouse	: Non Obese Diabetic mouse
OGTT	: Oral Glucose Tolerance Test
P2C	: Coxsackie Protein 2 C
PARP	: Poly-ADP ribose
PBMC	: Peripheral Blood Mononuclear Cells
PP	: Pancreatic Polypeptide
PPV	: Positive Predictive Value
RBA	: Radio Binding Assay
RIA	: Radio Immuno Assay
ROC	: Receiver Operated Curve
RR	: Relative Risk
SDS-PAGE	: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SMS	: Stiff Man Syndrome
Th-cell	: T helper lymphocyte
TNF	: Tumor Necrosis Factor
TRIGR	: Trial to Reduce Insulin dependent diabetes in the Genetically Susceptible
UKPDS	: United Kingdom Prospective Study
VP7	: Viral protein 7

6.2. Summary

Diabetes mellitus is a chronic disease characterised by hyperglycaemia. Glucose homeostasis is maintained by the hormones insulin and glucagon. Insulin is produced in the ß-cells in the islets of Langerhans in the pancreas. Two major forms of diabetes can be distinguished. Type-2 diabetes, the most common form of the disease, is caused by impaired insulin sensitivity and the inability of the islets of Langerhans to compensate for the increased insulin requirements. Destruction of the insulin producing ß-cells in the islets of Langerhans causes type-1 diabetes. This thesis addresses the question whether it is possible to identify people who will develop type-1 diabetes in the future.

In Chapter 1 a review on the pathophysiology and aetiology of type-1 diabetes and the currently available methods for diabetes prediction is given. Type-1 diabetes is the result of a gradual ß-cell destruction process. This process occurs sub-clinical, may last months to years and is often referred to as prediabetes. Type-1 diabetes will become clinically manifest when the majority of ß-cells has been destroyed, which may be early in youth, but type-1 diabetes may present in adults as well. The concept of prediabetes yields the unique opportunity to postpone or abolish clinical manifestation of type-1 diabetes by blocking ß-cell destruction. This requires identification of those at increased risk to develop type-1 diabetes.

ß-cell destruction in type-1 diabetes is autoimmune mediated. The immune system, which normally protects against foreign invaders like viruses or bacteria, considers ß-cells as foreign and destroys them. The exact cause of the destruction process is unknown, but it is clear that genetic and environmental factors play a role. Genetic factors lead to increased susceptibility for type-1 diabetes, but alone are not sufficient to cause type-1 diabetes. Additional environmental factors (diet, viral infections, vaccinations) are a prerequisite to initiate and maintain the destruction process. Only a small number of genetic factors that play a

role in type-1 diabetes have been identified today. Only 30% of the genetic susceptibility for type-1 diabetes can be explained by characterised genes, the most important of which is the HLA-region on chromosome 6. Some alleles in this HLA region confer a substantial increase in diabetes risk, while others confer dominant protection. Screening for genetic factors may be used as a first sieve to identify those at increased risk to develop diabetes, but will also identify a large number of individuals who will never contract type-1 diabetes (low specificity). Intervention based on such prediction methods will therefore result in unjust treatment of a large number of individuals.

Direct identification of the ß-cell destruction process may substantially increase specificity of screening. Detection of ß-cell autoimmunity is possible through the detection of autoantibodies to ß-cell antigens in the circulation. Islet cell antibodies (ICAs), insulin autoantibodies (IAA), antibodies to Glutamic Acid Decarboxylase (GAD) and antibodies against insulinoma antigen 2 (IA2) have been extensively studied for prediction purposes. The assays to detect ICAs ad IAAs are expensive, require large serum volumes and are, despite the organisation of international standardisation workshops, difficult to reproduce between different laboratories. On the contrary, the tests to detect GAD- and IA2-antibodies are highly reproducible and require small serum volumes. The practical work described in this thesis aims to evaluate the tests for GAD- and IA2-ntibodies and eventually develop a reliable prediction strategy for type-1 diabetes.

Basic knowledge on the natural course of the disease-process and the relation to antibody production is a prerequisite for the development of new preventive measures. Chapter 2 evaluates when in the disease process autoantibodies are formed, when they disappear and if they are continuously present. Our studies demonstrate that antibodies may remain present in the circulation up to 15 years after clinical manifestation of the disease. This is unexpected since it is generally

assumed that with disappearance of the ß-cells the immune system will be no longer stimulated to produce ß-cell antibodies. Another unexpected observation in our studies was that, in patients who are negative for antibodies at disease onset, antibodies may appear several years after diagnosis. It is not clear why these antibodies do appear, but apparently antibody formation is not correlated to the remaining ß-cell mass nor is there a correlation with ß-cell regeneration.

An important observation from the studies described in chapter 2 was that in first degree relatives of patients with type-1 diabetes antibodies may appear at any age. Although others confirm this observation, it is in contrast to the general assumption that ß-cell autoimmunity is initiated early in life. In addition, we described that first degree relatives of patients with type-1 diabetes may be transiently positive for GAD or IA2-antibodies. Both observations may have significant implications for diabetes prediction and warrant repeated serological testing. Single antibody-screening may both result in low sensitivity (missing those who later convert to seropositivity) or low specificity (identifying those who are transiently positive as high-risk individuals).

Additionally, we described that in first degree relatives, antibody levels may follow a seasonal pattern, being increased in spring. This increase coincides with the seasonal variation in enteroviral infections. Enteroviruses have been suggested to play a role in the pathogenesis of type-1 diabetes. Therefore we plan to study whether the increase in antibody levels is accompanied by viral infections in these first-degree relatives.

Most studies into the prediction of diabetes are performed in first degree relatives of probands with type-1 diabetes. The prevalence of the disease is increased in families and by confining prediction studies to family based populations fewer participants have to be included. However, most patients who contract the disease do not have a relative with type-1 diabetes. Consequently the incidence of type-1 diabetes can only be

effectively reduced when prediction and prevention can be applied in the general population. Chapter 3 evaluates if data on diabetes prediction obtained in family studies can be extrapolated to the general population. In a study of 1400 schoolchildren we demonstrated that this is indeed possible; of five children that tested positive for GAD- or IA2-antibodies two developed diabetes between one and eight years after serum sampling. Of those children that were negative for all antibodies none developed the disease.

Despite these promising results, several questions need to be answered. When extrapolating data between two populations it is important to take differences between the prevalence of the disease in these populations into consideration. The less prevalent a disease is, the lower the predictive value (the chance for an individual who tests positive to contract the disease) is. Consequently, prediction of type-1 diabetes in the general population requires an extremely high specificity of screening. In addition, it is not clear how to define positivity in antibody screening. The sensitivity and specificity of screening are strongly dependent on the threshold for positivity in an assay. Defining thresholds is ambiguous and there is a large variation in thresholds applied in different laboratories. Receiver operated Curve (ROC) analysis is widely applied to establish thresholds in medical practice, especially in diabetes research. The method finds a mathematical compromise between screening sensitivity and specificity. In GAD and IA2-antibody screening ROC analysis results in low thresholds and consequent low screening specificity. Since sera from prediabetic individuals are scarce, sera from newly diagnosed patients are used to establish screening sensitivity in ROC analyses. As demonstrated in chapter 2, and appeared from other studies as well, antibody levels (and properties) may significantly alter after clinical diagnosis. One may therefore wonder if the antibody data obtained in recent onset diabetes can be extrapolated to prediabetes. This phenomenon may partly explain the poor performance of ROC analysis described in chapter 3.

Type-1 diabetes is not just a disease of childhood, but may present in adults as well. The clinical manifestation in adults may be less acute than in children and it is generally assumed that this represents a slowly progressive form of type-1 diabetes, also referred to as latent autoimmune diabetes in adults (LADA). Since the onset of this type of diabetes closely resembles type-2 diabetes, the differential diagnosis may initially be hard to make. Patients are therefore often initially treated with diet or oral medication. After a period of several months, insulin injections are required to maintain glucose homeostasis. Earlier initiation of insulin therapy may be important to achieve sufficient glucoregulation to prevent onset of chronic microvascular complications. In addition, it has been reported that early initiation of insulin therapy may help to preserve remaining ß-cells, thus facilitating regulation of blood-glucose levels. Detection of ß-cell antibodies, GAD-antibodies in particular, is increasingly recognised as a useful tool to distinguish between type-1 and type-2 diabetes at disease onset.

In chapter 3 we used GAD-antibodies to estimate the prevalence of type-1 diabetes in a general population of elderly. In contrast to the prevalence of type-2 diabetes the frequency of type-1 diabetes appeared to be low. From studies performed in a hospital setting the prevalence of type-1 diabetes among patients initially diagnosed as type-2 diabetes approached 30%. In the population of elderly patients with diabetes described in chapter 3 the prevalence was much lower. This may be explained by patient selection. Since patients who have difficulties to maintain their glucose homeostasis are more often referred to the hospital for treatment. It is likely that these patients, who are more likely to have the slow-progressive form of type-1 diabetes, were over-represented in the hospital based studies.

Chapter 4 evaluates the technical aspects of GAD-antibody assays and possible confounding factors in predictive and diagnostic screening for type-1 diabetes. We demonstrated that GAD-antibody levels increase with age and that antibody levels are increased in females compared to males,

but differences did not affect threshold definition. In addition we studied whether diseases involving tissues that express the target of GAD-antibodies (Glutamic Acid Decarboxylase), lead to GAD-antibody formation. Antibody levels or frequencies were not increased in either of the diseases studied (epilepsy, cystic fibrosis, Guillain Barree syndrome and premature ovarian failure). A last important finding from these studies was that serum storage might affect antibody levels, which may have serious consequences for diabetes prediction studies.

Chapter 5 is a review of the results obtained by the practical work described in this thesis and the impact on diabetes prediction. Since there are currently no methods available for diabetes prediction, routine screening is useless and not recommended. In 2003 and 2005 the results of two large intervention trials will become available. If these studies prove to be effective it is important to have sensitive, specific and cheap screening strategies at hand. In addition, such strategies need to be available if new preventive measures are to be evaluated. The work described in this thesis has contributed to the improvement of the assays for GAD- and IA2-antibodies, and both assays are now applicable for large-scale predictive and diagnostic screening. A screening strategy based on genetic pre-selection and repeated serological screening seems technically feasible. However, additional data on the natural course of autoimmunity is required to improve screening sensitivity and specificity. In addition, studies on new predictive markers and refining analysis of existing may help to improve screening performance. Currently, we are working on the identification of new markers and studies on the dynamics of autoimmunity will be initiated.

6.3. Samenvatting

Diabetes mellitus (suikerziekte) is een chronische ziekte die wordt gekenmerkt door te hoge bloedsuikerspiegels. Normaalgesproken wordt de glucosehuishouding in het lichaam gereguleerd door een aantal hormonen waarvan insuline en glucagon de belangrijkste zijn. Deze hormonen worden geproduceerd in de Eilandjes van Langerhans die in de alvleesklier liggen. Er kunnen twee hoofdvormen van diabetes worden onderscheiden. De meest voorkomende vorm is type-2 diabetes (niet-insuline afhankelijke suikerziekte of ouderdomsdiabetes), die wordt veroorzaakt door een verminderde werking van insuline en onvermogen van de eilandjes van Langerhans om aan de daardoor verhoogde vraag naar insuline te voldoen. Bij type-1 diabetes (insuline afhankelijke suikerziekte of jeugd-suikerziekte) zijn de cellen die insuline maken vernietigd, hetgeen resulteert in een onvermogen om bloedsuikerspiegels op een normaal peil te handhaven. Daarnaast bestaan er nog een aantal minder vaak voorkomende vormen van diabetes die door verschillende factoren veroorzaakt worden. Dit proefschrift gaat over type-1 diabetes en de vraag of we kunnen zien aankomen wie er diabetes zal gaan krijgen.

Hoofdstuk 1 geeft een overzicht van de huidige kennis over de oorzaken van type-1 diabetes, de mechanismen die daarbij een rol spelen en de mogelijkheden die op dit moment beschikbaar zijn om diabetes te voorspellen en voorkomen. Type-1 diabetes wordt veroorzaakt door een geleidelijke afbraak van de insuline producerende ß-cellen. Dit afbraakproces verloopt voor de patiënt ongemerkt en kan maanden tot jaren duren. Naar deze fase wordt verwezen met de term prediabetes. Pas wanneer iemand bijna geen ß-cellen meer over heeft ontstaan er symptomen van type-1 diabetes. Dit kan op zeer jeugdige leeftijd gebeuren, maar ook bij volwassenen wordt vaak type-1 diabetes geconstateerd. Prediabetes geeft de unieke gelegenheid tot interventie in het ß-cel afbraakproces om zo het ontstaan van diabetes uit te stellen of zelfs geheel te voorkomen. Daarvoor is het echter nodig dat we mensen die

zich in deze fase bevinden kunnen identificeren – we moeten diabetes kunnen voorspellen.

De afbraak van de ß-cellen komt tot stand door een zogenaamd autoimmuunproces. Het afweersysteem (immuunsysteem), dat normaal-gesproken zorgt dat indringende virussen en bacteriën worden uitgeschakeld, ziet de ß-cellen aan voor vreemd en valt ze daarom aan. Waardoor dit afbraakproces veroorzaakt wordt is niet duidelijk, maar zeker is dat er een samenspel van erfelijke en omgevingsfactoren een rol speelt.

Erfelijke factoren geven een verhoogde vatbaarheid voor diabetes, maar niet iedereen met die hoge vatbaarheid zal ook daadwerkelijk de ziekte krijgen: er zijn omgevingsfactoren (dieet, virusinfecties, vaccinaties) nodig om het ziekteproces in gang te zetten. Bovendien is er relatief weinig bekend over de erfelijke factoren die een rol spelen. Slechts 30% van de erfelijkheid kan verklaard worden door voor ons bekende genen, waarvan de belangrijkste in het HLA-gebied op chromosoom 6 gelegen zijn. In dit HLA-gebied kennen we allelen die de vatbaarheid sterk verhogen, maar ook allelen die juist beschermen tegen type-1 diabetes. Onderzoek van erfelijk kenmerken kan helpen om mensen met een verhoogde of juist verlaagde kans op type-1 diabetes te identificeren, maar hiermee zullen ook mensen die de ziekte nooit zullen krijgen als hoog risico worden bestempeld (lage specificiteit). Wanneer er op basis van een dergelijke screeningsmethode een interventie zou worden gestart zouden er dus veel mensen voor niets worden behandeld.

Door daadwerkelijk het ziekteproces (de afbraak van de ß-cellen) op te sporen voordat iemand een tekort aan insuline krijgt, kan de specificiteit van screening verhoogd worden. Dit kan door het opsporen van antistoffen tegen ß-cellen in het bloed. De belangrijkste autoantistoffen die in dit proefschrift worden beschreven zijn eilandjesantistoffen (ICAs), insuline autoantistoffen (IAAs), antistoffen tegen glutaminezuur decarboxylase (GAD) en antistoffen tegen insulinoma antigeen 2 (IA2). De testen om ICAs en IAAs op te sporen zijn duur, er zijn grote hoeveelheden bloed voor

nodig en ze zijn, ondanks dat er internationale workshops georganiseerd worden voor standaardisatie van deze testen, slecht reproduceerbaar tussen verschillende laboratoria. De testen om GAD- en IA2-antistoffen op te sporen zijn daarentegen goed reproduceerbaar en er zijn maar kleine hoeveelheden bloed nodig om een kwantitatieve bepaling te doen. Het werk beschreven in dit proefschrift heeft tot doel deze tests nader te evalueren om zo een betrouwbare methode voor de predictie van type-1 diabetes te ontwikkelen.

Om goede predictie strategieën te kunnen ontwerpen is kennis van het natuurlijk beloop van het ziekteproces en de wijze waarop dit tot uiting komt in de productie van autoantistoffen noodzakelijk. In hoofdstuk 2 is onderzocht wanneer antistoffen voor het eerst ontstaan, waneer ze weer verdwijnen en of ze continue in het bloed aantoonbaar zijn. Het is gebleken dat ß-cel antistoffen nog lange tijd (tot 15 jaar) na diagnose in het bloed van patiënten aantoonbaar kunnen zijn. Dit is verassend omdat algemeen wordt aangenomen dat met het verdwijnen van de laatste ß-cellen het afweersysteem niet meer gestimuleerd zal worden om antistoffen aan te maken. Veel verrassender nog was onze observatie dat patiënten die bij de diagnose van diabetes geen antistoffen in het bloed hebben, deze later alsnog kunnen vormen. De oorzaak hiervan is onbekend, de aanmaak van de antistoffen lijkt niet samen te hangen met nog resterende ß-cellen en ook niet veroorzaakt te worden door nieuwe aanmaak van deze cellen.

Een andere belangrijke observatie in deze studie was dat bij familieleden van patiënten met type-1 diabetes antistoffen op ieder moment in het leven kunnen ontstaan. Hoewel deze observatie ook door anderen wel is beschreven, staat het tegenover de algemene aanname dat autoantistoffen al vroeg in het leven ontstaan. Daarnaast bleek dat bij verwanten van patiënten met diabetes soms tijdelijk ß-cel antistoffen aantoonbaar waren in het bloed. Beide bevindingen hebben consequenties voor diabetes predictie. Ze geven aan dat eenmalige screening op ß-cel antistoffen onvoldoende is omdat hiermee zowel mensen die later alsnog antistoffen

(en diabetes) ontwikkelen gemist kunnen worden (lage sensitiviteit – het percentage van de mensen die diabetes krijgen die positief zijn in de test), als ook mensen die slechts tijdelijk positief zijn kunnen worden beschouwd als hebbende een hoog risico (lage specificiteit).

Een derde belangrijke bevinding van het onderzoek beschreven in hoofdstuk 2 was dat antistof spiegels bij eerstegraads familieleden fluctueerden door het jaar, waarbij in het voorjaar de hoogste antistof spiegels werden gemeten. Deze piek in antistofspiegels valt samen met het seizoen waarin veel infecties met het enterovirussen plaatsvinden. Dergelijke infecties verlopen meestal asymptomatisch, maar enterovirussen worden in veel onderzoeken gezien als een belangrijke omgevingsfactor die diabetes kan veroorzaken. Deze bevindingen vormen dan ook de aanleiding voor verder onderzoek naar enterovirus-infecties bij diegenen die fluctuaties in antistof spiegels vertoonden.

Veel onderzoek naar het voorspellen van type-1 diabetes wordt uitgevoerd bij familieleden van patiënten. De ziekte komt bij familieleden vaker voor dan in de algemene bevolking en door predictieonderzoek te beperken tot deze groep hoeven minder mensen in de studies te worden betrokken. Echter, meer dan 90% van de mensen die de ziekte krijgen hebben géén familielid met type-1 diabetes. Diabetes kan daarom alleen op grote schaal worden voorkomen wanneer we het in de algemene populatie kunnen voorspellen en voorkomen. In hoofdstuk 3 wordt onderzocht of de gegevens over diabetes predictie die verzameld zijn in familieonderzoek, naar de algemene populatie kunnen worden geëxtrapoleerd. In een onderzoek van 1400 schoolkinderen hebben we aangetoond dat dit mogelijk is; van de vijf kinderen die positief waren voor GAD en/of IA2-antistoffen kregen er twee diabetes tussen één en acht jaar na het onderzoek. Van de kinderen die geen antistoffen hadden kreeg er niet één diabetes.

Ondanks deze hoopgevende resultaten zijn er nog een aantal problemen die moeten worden opgelost. Bij extrapolatie van gegevens tussen twee

populaties dient rekening te worden gehouden met het verschil in de frequentie van de ziekte in die populaties. Hoe minder vaak een ziekte voorkomt in een populatie hoe lager de voorspellende waarde (de kans dat iemand ziek wordt wanneer hij een positieve testuitslag heeft) van een positieve test is (regel van Bayes). Voor predictie in een populatie met een lage ziektefrequentie is daarom een test nodig met zeer hoge specificiteit. Daarnaast is het niet duidelijk bij welke waarden de uitslagen van de GAD en IA2-antistof bepalingen als positief beschouwd moeten worden. De sensitiviteit en specificiteit van een test hangen zeer nauw samen en worden voor een groot deel bepaald door de drempelwaarde voor positiviteit die in een test gehanteerd wordt. Het definiëren van een drempelwaarde is arbitrair en er worden verschillende drempelwaarden gehanteerd in verschillende laboratoria. In hoofdstuk 3 wordt gedemonstreerd dat een veel gebruikte methode voor het definiëren van drempelwaarden, de Receiver Operated Curve analyse (ROC), die een mathematisch compromis vindt tussen sensitiviteit en specificiteit, leidt tot een lage drempelwaarde die veel vals positieve testuitslagen (lage specificiteit) tot gevolg heeft. Bij de ROC-analyse wordt voor het vaststellen van de sensitiviteit van de test gebruik gemaakt van de antistofspiegels verkregen in nieuw gediagnostiseerde patiënten. Uit de studies die in hoofdstuk 2 zijn beschreven, en ook uit andere studies, is gebleken dat er na het ontstaan van de ziekte veranderingen optreden in antistoffen. Het is daarom de vraag of de gegevens verkregen in nieuw gediagnostiseerde patiënten wel van toepassing zijn op de prediabetische fase. Dit fenomeen kan mogelijk de slechte prestatie van de ROC analyse gedeeltelijk verklaren.

Type-1 diabetes is niet alleen een kinderziekte, maar kan zich ook op hogere leeftijd manifesteren. Er wordt algemeen aangenomen dat het hier om een langzaam progressieve vorm van type-1 diabetes gaat, ook wel diabetes type-1½ genoemd. De symptomen bij volwassenen zijn vaak minder acuut dan bij kinderen en vaak wordt dan ook ten onrechte de

diagnose type-2 diabetes gesteld. Na een periode van behandeling met dieet of tabletten moeten deze patiënten echter toch overschakelen op insuline injecties om de een goede regulatie van bloedsuikerspiegels te kunnen handhaven. Het kan van belang zijn om al eerder met insulinetherapie te beginnen om een betere glucosehuishouding te handhaven, hetgeen van belang is om late complicaties van diabetes (nierziekten, hart en vaatziekten, oogaandoeningen en zenuwaandoeningen) te voorkomen. Bovendien wordt gesuggereerd dat, door vroeg met insuline injecties te beginnen, de resterende ß-cellen gespaard kunnen blijven, hetgeen de regulatie van de bloedsuikerspiegels ten goede komt. ß-cel autoantistoffen, in het bijzonder antistoffen tegen GAD, kunnen van grote waarde zijn om al vroeg de juiste differentiaal diagnose te stellen. Zij worden dan ook in toenemende mate als aanvullend diagnostisch middel gebruikt. In hoofdstuk 3.3 is door screening voor GAD-antistoffen onderzocht hoe vaak type-1 diabetes voorkomt in een algemene oudere populatie. De frequentie blijkt zeer laag te zijn (in tegenstelling tot type-2 diabetes). Uit vroegere studies van patiënten met diabetes die werden behandeld door de internist werd geconcludeerd dat tot 30% van de patiënten waarbij oorspronkelijk de diagnose type-2 diabetes was gesteld, mogelijk type-1 diabetes had. In onze studie van de algemene oudere populatie bleek dit percentage veel lager te zijn, hetgeen waarschijnlijk verklaard kan worden door de selectie van de patiënten die werden onderzocht. Bij de internist worden voornamelijk patiënten met een moeilijk te reguleren type-2 diabetes behandeld en juist hieronder zullen zich veel patiënten met type-1 diabetes bevinden.

In hoofdstuk 4 wordt ingegaan op de technische specificaties van de test voor GAD-antistoffen en mogelijke factoren die prestatie van de test kunnen beïnvloeden, voor zowel diagnostische als predictieve toepassingen. In een studie van de algemene bevolking werd aangetoond dat GAD antistof spiegels toenemen met toenemende leeftijd, maar dat

deze toename zo minimaal is dat het niet nodig is om de drempelwaarden voor positiviteit hieraan aan te passen. Daarnaast is er aangetoond dat vrouwen iets hogere antistof spiegels hebben dan mannen, maar dat ook deze bevinding is niet van belang voor de definitie van drempelwaarden. Bovendien, werd er onderzocht of bepaalde ziekten waarbij weefsels waarin de het doelwit van GAD-antistoffen (glutamaat decarboxylase) geproduceerd wordt zijn betrokken, aanleiding kunnen geven tot de productie van GAD-antistoffen. Bij geen van de ziekten die werden onderzocht; epilepsie, cystische fibrose (taaislijmziekte), Guillain Barré syndrome en premature ovarian failure, werd een verhoogde frequentie van GAD-antistoffen aangetroffen. Een laatste belangrijke bevinding uit het onderzoek beschreven in hoofdstuk 4 was dat de wijze van opslag van serum van invloed kan zijn op de gemeten antistof spiegels, hetgeen van groot belang kan zijn voor diabetes predictie-studies.

Hoofdstuk 5 geeft een overzicht van de resultaten behaald met het praktisch werk dat in het proefschrift is beschreven en de consequenties voor diabetes predictie. Omdat er nog steeds geen methoden voor preventie van type-1 diabetes beschikbaar zijn is routinematige screening op dit moment overbodig en niet wenselijk. Echter in 2003 en 2005 worden de resultaten van twee grote interventie trials verwacht. Mocht er uit deze onderzoeken blijken dat diabetes preventie mogelijk is dan is het van groot belang dat er een sensitieve, specifieke, goedkope en praktisch toepasbare screenings-strategie voorhanden is. Daarnaast zijn dergelijke screenings-strategieën noodzakelijk voor het opzetten van nieuwe interventie-studies. Het werk beschreven in dit proefschrift heeft bijgedragen aan de verfijning van de testen voor GAD- en IA2-antistoffen en beide testen zijn nu praktisch toepasbaar voor bevolkingsonderzoek. Een predictie-strategie bestaande uit een voorselectie op basis van genetische merkers, gevolgd door herhaaldelijke antistof tests is denkbaar. Er zijn echter nog te veel lacunes in de kennis over het natuurlijk beloop van ß-cell autoimmuniteit om tot een betrouwbare predictie-strategie te komen.

Nader onderzoek naar nieuwe predictieve merkers en verfijning van bestaande technieken is daarom noodzakelijk. Momenteel wordt er daarom binnen de diabetes groep van de Earsmus Universiteit en het Sophia Kinderziekenhuis gewerkt aan de identificatie van nieuwe antigenen en nadere beschrijving van de dynamiek van autoimmuniteit.

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Veel van de publicaties in dit boekje zijn tot stand gekomen met de steun van medisch studenten die hun afstudeerstage aan diabetes predictie hebben gewijd. Hierbij wil ik speciaal *Maarten, Frank, Arianne, Sabine en Manda* noemen. Ik vond het leuk om met jullie samen te werken! Succes met jullie verdere opleiding en carriere!

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Publications

M. Ledeboer, AAM Masclee, M.R. Batstra, JBMJ Jansen, CBHW Lamers. Effect of cholecystokinin on lower esophageal sphincter pressure and transient lower esophageal sphincter relaxations in man. *Gut 1995;36:39-44*

MR Batstra, HJ Aanstoot, GJ Bruining. Etiologie en pathogenese van diabetes mellitus type I. In: Diabetes Mellitus. Editors: E van Ballegooie, RJ Heine. Utrecht 1995.

MR Batstra, M Pina, J Quan, P Mulder, CE de Beaufort, GJ Bruining, HJ Aanstoot. Fluctuations in GAD_{65}-antibodies after clinical diagnosis of insulin-dependent diabetes mellitus in young children. *Diabetes care 20 (4): 642-644, 1997*

JB Ruige, MR Batstra, HJ Aanstoot, LM Bouter, GJ Bruining, JND de Neeling, RJ Heine. Low prevalence of antibodies to GAD65 in a 50 to 74 year-old general dutch population. *Diabetes Care 20 (7): 1108-1110, 1997*

MR Batstra, GJ Bruining, HJ Aanstoot. Antibody screening in a population of children. *Annals of medicine 29 (5): 453-460, 1997*

MR Batstra. Diabetes predictie en preventie: gaat type I diabetes de wereld uit? *Nederlands tijdschrift voor kindergeneeskunde 66 (6): 249 – 255, 1998*

NC Schloot, MR Batstra, G Duinkerken, RRP de Vries, T Dyrberg, A Chaudhurri, PO Behan, BO Roep. Regulation of GAD65 reactive T-cells in a non-diabetic SMS patient. *Journal of autoimmunity 12 (4):289-296*

GR Vreugdenhil, MR Batstra, HJ Aanstoot, WJG. Melchers, JMD. Galama. Analysis of antibody responses against non-structural coxsackie B virus proteins in patients with type-1 diabetes. *In press*

GJ Bruining, MR Batstra. Recent advances in the immunology of type 1 diabetes mellitus: a congenital disorder? *In press*

MR. Batstra, A van Driel, JS Petersen, CA van Donselaar, MJ van Tol, GJ Bruining, DE Grobbee, T Dyrberg, HJ Aanstoot.GAD-antibodies in screening for autoimmune diabetes – influence of age. Sex and other diseases on specificity and assay thresholds. *Submitted*

MR Batstra, JS Petersen, AM Verwest, S de Man, JL Molenaar, DE Grobbee, GJ Bruining, T Dyrberg, HJ Aanstoot. Prediction of type I diabetes mellitus in schoolchildren – threshold definition and assay conditions. *Submitted*