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## Immune therapy in type 1 diabetes mellitus.

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## **Title: Immune therapy in type 1 diabetes mellitus**

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### **Competing interests**

Å. Lernmark declares associations with the following companies: Diamyd Medical AB, Stockholm, Sweden and Zealand Pharma, Copenhagen, Denmark. H. E. Larsson declares no competing interests.

**Abstract** (200 words) Type 1 diabetes mellitus (T1D) is an autoimmune disorder directed against the pancreatic islet  $\beta$  cells. The genetic risk for the disease is linked to HLA-DQ genotypes and unknown environmental triggers. In most countries, only 10-15% of newly diagnosed T1D children or young adults have a first degree relative with the disease. Autoantibodies against insulin, GAD65, IA-2 or ZnT8 transporter mark islet autoimmunity. These islet autoantibodies may develop already at 1-2 years of age. Immune therapy in T1D is approached at three stages. Primary prevention is treatment of subjects at increased genetic risk. The TRIGR trial is testing if hydrolyzed casein milk formula may reduce T1D in genetically predisposed infants. Secondary prevention is in subjects with persistent islet autoantibodies. On-going trials are either non-autoantigen (BCG, CD3 monoclonal antibodies) or autoantigen (oral and nasal insulin or Alum-formulated GAD65) specific. Intervention at diabetes onset include non-autoantigen (CD3 monoclonal antibodies, IL-2 receptor antibodies, IL-1b receptor inhibitor, IL-1b antibodies, BCG, ATG, DiaPep277) and autoantigen (proinsulin peptides) specific therapy. Although preserved beta-cell function long term has been difficult to achieve in many prior studies, considerable progress is being made through controlled clinical trials and animal investigations to uncover mechanisms of beta-cell destruction. Novel therapies that would prevent islet autoimmunity or halt progressive beta-cell destruction need to be designed.

**Key points (4-6 sentences)**

The evidence that type 1 diabetes is an autoimmune disease is the association to HLA-DQ and the role of environmental factors. It is of interest that the majority of non-HLA genes contributing to disease risk are all related to the function of the immune system.

Autoantibodies to the beta cell autoantigens insulin, GAD65, IA-2 and ZnT8 transported are major markers of islet autoimmunity. The number of islet autoantibodies determines risk and time to clinical onset of diabetes.

The approach to immune therapy follows three levels: primary prevention, secondary prevention and intervention.

Primary prevention is based on the possibility to identify subjects at risk already at birth. Induction of immunological tolerance to islet autoantigens is a goal but difficult to measure.

Secondary prevention is in subjects who have developed persistent islet autoantibodies. This intervention is either non-autoantigen or autoantigen specific. Combination therapies have yet to be carried out.

Intervention is to randomize subjects with recently diagnosed type 1 diabetes. The most common primary outcome in clinical studies and trials is to preserve c-peptide. Both non-autoantigen and autoantigen specific therapies are carried out.

**Biographies**

Åke Lernmark

Helena Elding Larsson

**Breakdown of proposed sections**

1. Background
2. Primary prevention
  - a. Non-autoantigen specific
  - b. Autoantigen specific
3. Secondary prevention
  - a. Non-autoantigen specific
  - b. Autoantigen specific
4. Intervention

- a. Non-autoantigen specific
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- 5. Future directions
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## 1. Background

Type 1, or autoimmune diabetes (T1D) is characterized by a complete immune-mediated specific destruction of the pancreatic islet  $\beta$  cells. The autoimmune destruction is chronic and continuous well after the clinical diagnosis when some residual beta-cell function may still be detected. Eventually, after some 2-3 years of insulin therapy, essentially all  $\beta$  cell are destroyed resulting in an almost complete inability to produce insulin. Hence, the disease is fatal as it is not possible to live without insulin. All patients with T1D therefore take daily insulin injections to survive. The replacement therapy is inadequate since it is next to impossible to obtain a perfect balance between the insulin injected and the actual need for the hormone. Additionally, in contrast to the secreted insulin, subcutaneous injections of insulin, do not permit insulin to reach the liver directly. The liver is a first target organ for insulin secreted from the  $\beta$  cells. However, the current mode of insulin administration does not accomplish this task.

Ever since the discovery of insulin in 1921, it has been possible to keep patients alive with insulin replacement therapy. It has been very important that the replacement therapy has been evolving continuously as at present there is no other treatment that a newly diagnosed T1D patient can be offered. Insulin analogues with different times of action, long-acting as well as short or rapid acting insulin products have been on the market for several years (reviewed in <sup>1</sup>). These analogues combined with novel approaches to administration and glucose control will undoubtedly increase the quality of life for thousands of patients. Continuous glucose monitoring and improved devices for blood glucose testing has contributed to better routes and means of administration. These approaches to replacement therapy will improve blood glucose control and diabetes control overall. However, none of the approaches to control diabetes with insulin analogues are addressing the underlying cause of T1D. It is therefore of considerable importance that controlled clinical trials are designed to interfere with either the etiology or the pathogenic process that eradicates the  $\beta$  cells. Current understanding of the etiology and pathogenesis of T1D allow the design of both primary and secondary intervention trials. The approach to T1D prevention and intervention clinical trials rests on three factors: 1) *genetic etiology*; susceptibility to islet autoimmunity and T1D is inherited. Subjects at genetic risk for islet autoimmunity would be treated in a primary prevention approach. The objective would be to secure or induce immunological tolerance to islet autoantigens; 2) *islet autoimmunity* is marked by the appearance of autoantibodies against specific autoantigens. Subjects developing islet autoimmunity would be treated in a secondary prevention approach. The objective would be to prevent the loss of  $\beta$  cells either by inducing immunological tolerance to one or several islet autoantigens or inhibiting the autoimmune process, or both; and 3) *c-peptide* as measure of residual  $\beta$ -cell function. Subjects who have lost a sufficient number of  $\beta$  cells or  $\beta$ -cell function would be offered intervention therapy. Also here the objective would be to prevent the loss of  $\beta$

cells either by inducing immunological tolerance to one or several islet autoantigens or inhibiting the anti- $\beta$ -cell autoimmune process, or both.

The *genetic etiology* is strongly linked to HLA on the short arm of chromosome 6<sup>2</sup>. There are two extended HLA haplotypes, which confer a marked increased risk for T1D (Figure 1). Among Caucasians, the extended HLA haplotypes DRB1\*04-DQA1\*03:01-B1\*03:02 (DR4-DQ8) and DRB1\*03:01-DQ A1\*05:01-B1\*02:1 (DR3-DQ2) alone or in combination may be present in nearly 90 % of T1D patients diagnosed before 18 years of age. As many as 27-30 % of patients have the DR4-DQ8/DR3-DQ2 heterozygous genotype compared to 3.5 % among newborn children. The risk for T1D is further increasing if there is a family member with T1D. The lifetime risk for having a sibling is about 8%, a father with T1D about 5% and a mother about 3%. Rapid onset T1D in children younger than 3-4 years of age happens particularly often in children carrying the DR4-DQ8/DR3-DQ2 heterozygous genotype. HLA typing alone may therefore be one possible approach to identify individuals for primary prevention trials. However, as only a small fraction of such subjects (in Sweden 3.5% of newborns are born with this genotype but only 6/100 will develop T1D in a lifetime) it may be important to increase the odds for islet autoimmunity or diabetes by adding non-HLA genetic factors to the inclusion criteria.

Genome wide association studies (GWAS) of T1D patients and compared to controls have identified more than 40 genetic factors that may be related to T1D risk<sup>3</sup>. These formidable investigations underscore the unique risk conferred by HLA. Only INS and possibly PTPN22 contributes to risk to the extent that it would be a must to take these genetic factors into account when selecting individuals for clinical trials to prevent T1D. The contributions of the remaining 50+ loci for either islet autoimmunity, T1D, or both remains to be determined.

*Islet autoimmunity.* While HLA is the strongest marker of the genetic etiology, islet autoantibodies are the strongest and currently the only available markers of islet autoimmunity and therefore of disease pathogenesis (Figure 1). Currently T1D is predicted by autoantibodies against the following four beta-cell proteins: insulin, GAD65, IA-2 and the ZnT8 transporter (reviewed in<sup>4,5</sup>). GADA, IA-2A and ZnT8A appear robust and are standardized in inter-laboratory round robin exercises through first the Immunology of Diabetes Workshops<sup>6</sup> and lately by the Diabetes Autoantibody Standardization Program (DASP)<sup>7</sup>. Only IAA remains to be fully standardized<sup>8</sup>. The Immunology of Diabetes Society has begun efforts to harmonize and standardize cellular assays for islet autoimmunity including both HLA class I and II restricted T cell activities (Malone et al 2011). As both clinical studies and trials are multicentre investigations, it is becoming more and more critical to standardize and harmonize also assays detailing cellular responses to treatment, Analyses of islet autoantibodies in the general population, among parents or siblings to patients with T1D makes it possible to randomize subjects with more than one islet autoantibody into secondary prevention clinical trials to delay or prevent T1D. It is well documented in TrialNet-studies of subjects with T1D first-degree relatives that the presence of a single islet autoantibody only marginally increases the risk for T1D<sup>4</sup>. However, the risk for T1D increases with an increasing number of islet autoantibodies (Figure 2). While 50% of subjects who have or have had two islet autoantibodies may develop T1D over five years, it will only take 3 years to get the disease for 50% of subjects who have more than three islet autoantibodies.

Prospective follow-up of subjects at risk for the development of persistent islet autoantibodies is therefore an effective approach to randomize individuals into secondary prevention trials.

The identification of individuals with islet autoantibodies makes it possible to follow these subjects until diabetes is diagnosed to make observations on the natural history of the disease. This is less satisfactory than to be able to ask such individuals to participate in secondary prevention trials. Is there any benefit for an islet autoantibody-positive child or young adult to be followed only to observe the inevitable loss of beta-cell function? Recent investigations in both the DAISY<sup>9</sup> and the TEDDY<sup>10</sup> studies demonstrate that diabetes is often diagnosed in observational clinical studies without the classical symptoms of T1D. Hence, at the time of clinical diagnosis of diabetes the patients may still have a significant beta-cell reserve and would therefore benefit from low insulin requirements compared to patients who are diagnosed in ketoacidosis and with a major loss of endogenous insulin production. An early diagnosis of diabetes may therefore change the outcome of intervention trials<sup>9</sup>.

*C-peptide*.. The loss of c-peptide as a proxy for residual  $\beta$  cells is significant already at the time of clinical diagnosis. Although reduced in an age-dependent fashion to levels well below the normal range, it has been feasible reliably to measure c-peptide and test if a reduction in the decrease in c-peptide was possible despite the loss with increasing duration of the disease<sup>11</sup>. The possibility to randomize subjects into intervention clinical trials when they have significant residual  $\beta$ -cell function – often c-peptide within the normal range in young adults or adults - may improve the outcome in intervention clinical trials. Ideally, the patients should need less insulin to control their blood glucose and maintain a normal HbA1c. So far, none of the many intervention trials in T1D has achieved a reduction long-term in c-peptide disappearance rate. Some trials have achieved promising results but in most trials the reduction in c-peptide disappearance has been transient. Although these clinical trials have improved our understanding of the disease pathogenesis, there is a need for profoundly different approaches to achieve significant and long-lasting prevention of  $\beta$ -cell disappearance in T1D. In this review, we summarize recent and on-going clinical studies and trials based on the staging of autoimmune diabetes i.e. primary prevention, secondary prevention and intervention. The reader is referred to recent reviews and other perspectives on clinical trials in T1D (ref,ref).

Prevention Implementation Coordination Centre for T1D United Research in Europe (PICCTURE) (<http://www.ludc.med.lu.se/research-units/diabetes-and-celiac-disease/research-projects/diabetes-type-1-prediction-early-pathogenesis-and-prevention/picpicture-activities/>) provides an updated listing of on-going prevention clinical trials in subjects with islet autoantibodies. The listing includes whether the study is primary, secondary or tertiary (intervention) prevention trial. Tables include summary of the latest information about the progress in these trials with links to the official webpage of respective trial on both the EudraCT (<https://eudract.ema.europa.eu/>) and clinicaltrials.gov (<http://clinicaltrials.gov/>) websites.

## 2. Primary prevention – treating subjects at genetic risk for islet autoimmunity or type 1 diabetes.

Primary prevention trials in T1D are quite conjectural at present. As triggers of islet autoimmunity have not been identified we can only guess what to treat in order to prevent islet autoimmunity. Any trial would aim at hindering triggers of islet autoimmunity in subjects with increased genetic susceptibility for T1D. A current weakness is that islet autoantibodies i.e. insulin, GAD65, IA-2 and ZnT8 transport IgG are the only marker for islet autoimmunity. Antigen-presenting cells, T and B cells responses that are expected to precede the autoantigen IgG are yet to be discovered. The approach to primary prevention would either be non-autoantigen or autoantigen-specific. A non-autoantigen specific therapy would remain speculative as long as a trigger of islet autoimmunity has not been identified. An autoantigen-specific therapy would address the question whether immunological tolerance against islet autoantigens may be induced early in life. Factors such as the duration of the intervention, the stage of enrollment and drug dosage and safety, would remain critical to a successful outcome. There are two major problems with primary prevention. First, there is at present no accepted method to determine whether an individual has developed immunological tolerance to an autoantigen. In other words, what would be the approach to find out if there is a “hole” in the immunological repertoire at about 1-2 years of age indicating a risk to mount a T and B cell mediated immune response to islet autoantigens? Second, immune tolerance induction may be safe but what would be the approach to find out if the treatment has been successful?

### a. Non-autoantigen primary prevention

Early exposure to cow's milk protein is hypothesized to increase the risk for T1D<sup>12</sup>. Dietary manipulation using hydrolyzed casein milk formula showed promise to reduce the risk for islet autoimmunity and T1D<sup>13</sup>. The TRIGR trial is an international effort involving 17 countries to test if hydrolyzed casein milk formula may reduce T1D in genetically predisposed infants born in families with T1D. Following a period of 6-8 month of breast-feeding, infants were randomized into either receiving hydrolyzed casein-based or conventional cow's milk formula<sup>12</sup>. The randomization code will be opened when the last recruited child turns 10 years of age, which is not until 2017 (ref 13)

It has been speculated that vitamin D protects against islet autoimmunity, T1D, or both<sup>15</sup> possibly through effects on T lymphocytes<sup>16</sup>. Lower concentrations of vitamin D have been reported in T1D children and lower vitamin D levels during pregnancy were suggested to increase the risk for T1D<sup>17</sup>.

Supplementation with cod liver oil, an important source of vitamin D and omega-3 fatty acids, during the first year of life led to reduced risk of T1D in Norwegian children, but no risk reduction was found with other kinds of vitamin D supplementation, suggesting that omega-3 fatty acids were responsible for the effect<sup>18</sup>. A clinical trial in children at increased susceptibility for islet autoimmunity, T1D, or both with cod liver oil would be possible.

The Nutritional Intervention to Prevent Diabetes (NIP-Diabetes) is a pilot study to test a proposed preventive effect of oral docosahexanoic acid (DHA) against islet autoimmunity (trial identifier NCT00333554)<sup>19</sup>. This study is ongoing with pregnant mothers in their 3<sup>rd</sup> trimester who had HLA-risk DQ types and had T1D themselves or



a family history of T1D. DHA has been taken in late pregnancy and early infancy and the infants are followed for the development of islet autoantibodies<sup>19</sup>. The NIP study itself is not powered to detect an effect on the development of T1D.

Taken together, it is feasible to screen newborns for HLA<sup>14,20</sup>. Children with increased susceptibility for islet autoimmunity are easily identified. In non-antigen primary prevention trials, it would seem important to select children with genetic susceptibility (HLA-DQ typing of cord blood is an effective screening method) for islet autoimmunity to limit overtreatment. We believe that the outcome of the TRIGR study will be guiding future attempts of non-autoantigen primary prevention.

#### b. Autoantigen primary prevention.

Autoantigen-specific primary prevention would address the question whether immunological tolerance induced against insulin, GAD65, IA-2 or ZnT8 would prevent the appearance of autoantibodies to these autoantigens. In order to avoid overtreatment it would be required that children with high genetic risk for islet autoimmunity or T1D are first identified. Selecting children with the HLA-DQ 2/8 genotype would represent about 3-4% of all newborns but 27% of all children diagnosed with T1D before 18 years of age. HLA-DQ 2/8 heterozygous children born in families with a T1D mother, father or sibling would increase the risk further and is likely to be associated with an earlier age at onset of islet autoimmunity.

The proposed treatment in this group of children would have to have a very high safety profile as only a small fraction of the children would develop islet autoimmunity, T1D, or both.

One primary prevention trial, Pre-POINT or Primary intervention with oral insulin for prevention of T1D is underway in infants with high genetic risk to develop diabetes<sup>21</sup>. The rationale is to use insulin in an attempt to induce immunological tolerance in order to prevent the appearance of insulin autoantibodies (IAA). Pre-POINT is ongoing and both oral and nasal insulin will be tested in genetically predisposed infants aged 18 months to 7 years with no islet autoimmunity. The children are born in a family with at least one member affected by T1D. Pre-POINT will be testing oral insulin at a dose almost 10 times that of the failed DPT-1 trial<sup>22</sup>. Although the Pre-POINT study is focused on a particular very high-risk group of children (HLA-DQ2/8 children born to T1D mothers), colleagues were skeptical. Ethical concerns were raised along with the lack of understanding of future consequences of exposing infants not only to mucosal insulin but also to this type of randomized, controlled clinical trial.

### **3. Secondary prevention – treating subjects who have developed islet autoantibodies.**

Secondary prevention trials have been carried out for the past 20 years with little success in terms of prevention but with a major advance in demonstrating that major undertakings are possible in addition to novel observations that help to understand the disease process in children who have developed islet autoantibodies. The Diabetes Prevention Trial Type 1 (DPT-1) recruited relatives of patients with T1D throughout the United States and Canada<sup>22</sup>. The DPT-1 trial has provided fundamental understanding of the progression of islet autoantibody-positive subjects to T1D clinical onset (Figure 2). The larger the number of islet autoantibodies, the shorter the time to clinical diagnosis of diabetes. Additional secondary prevention trials are

feasible as both efficient HLA-DR-DQ typing and standardized islet autoantibody tests are available. HLA typing makes it possible to confirm that a subject to be randomized into a secondary prevention trial has a stipulated genetic risk for T1D. For example, subjects with the HLA-DQ A1\*01:02-B1\*06:02 haplotype may be excluded because this haplotype is rarely seen among T1D patients younger than 12 years of age. Future investigation may need to take into account islet autoantibodies are related to HLA-DQ. For example, HLA-DQ8 is associated with an increased risk to develop diabetes with IAA, IA-2 or ZnT8A, DQ2 is associated with an increased risk for GADA but decreased for IA-2A and DQ6.4 is associated with ZnT8A (ref). In the secondary prevention approach, it is asked whether immunomodulating agents, representing either a non-autoantigen specific treatment or treatment with any of the four specific islet autoantigens would halt the progression to clinical onset of diabetes in islet autoantibody-positive individuals.

a. Non-autoantigen secondary prevention.

Non-antigen-specific agents were tested in a number of secondary prevention trials. Cyclosporine was given to ICA-positive first-degree relatives to T1D patients. The treatment did not reduce the progression to clinical onset<sup>23</sup>. BCG vaccine was given to a group of subjects with islet autoimmunity<sup>24</sup>. The hypothesis was that this treatment, associated with a marked non-specific stimulation of the immune system would halt progression to T1D. No evidence was found that BCG vaccination could prevent against  $\beta$ -cell-damaging processes leading to T1D in genetically at-risk children. Ketotifen was administered to islet autoantibody-positive subjects<sup>25</sup>. It was tested if the antiedematous therapy with this histamine antagonist would preserve  $\beta$ -cell function. The treatment did not induce protection<sup>25</sup>. Nicotinamide was tested in two large clinical trials, ENDIT<sup>26</sup> and DENIS<sup>27</sup>. Based on animal studies, nicotinamide was thought to halt progression to T1D. The outcome of these two trials showed that the treatment had no effect. Finally, gluten-free diet was tested as gluten-exposure was thought to modulate the risk for islet autoantibodies among first degree relatives to T1D patients<sup>28, 29</sup>. In the BABYDIET<sup>30</sup> studies, gluten-free diet was given to islet autoantibody-positive children without any significant preventive effect on the risk of T1D.

b. Autoantigen-specific secondary prevention

It was suggested that insulin therapy in individuals with islet autoimmunity would be advantageous for two reasons. The first would be that insulin would reduce the  $\beta$ -cell load in the state of subclinical diabetes. The second would be the possibility that also immunomodulatory effects could not be excluded. The treatment with insulin was tested as either parenteral, oral or intranasal. Evidence of delaying disease progression was obtained in pilot studies, which tested parenteral insulin (subcutaneously and intravenously) as prophylaxis among T1D first-degree relatives with islet cell antibodies (ICA)<sup>31</sup>. In retrospect, this pilot was complicated by the fact that some of the participants (delayed onset) had protective HLA genotypes<sup>32</sup>.

*Parenteral insulin.*

More than 80,000 relatives were screened for ICA. The intervention consisted of low-dose subcutaneous ultralente insulin, administered twice daily for a total dose of 0.25 unit per kilogram of body weight per day. At this dose, there was no delay or

prevention of T1D<sup>22</sup>. The randomized and controlled Diabetes Prevention Trial-1 (parenteral arm) therefore failed to reproduce the results of the pilot studies. As only one dose of insulin was tested and the subjects already showed reduced  $\beta$ -cell function at the time of randomization it was not possible to answer the question whether the insulin had any effect on protecting the  $\beta$ -cells, inducing immunomodulation, or both. Nevertheless, the DPT-1 trial pointed at the feasibility of screening for subjects with T1D genetic susceptibility and islet autoimmunity. The screening program continues through TrialNet (<http://www.diabetestrialnet.org/researchers/index.htm>).

#### *Oral insulin.*

ICA and IAA positive DPT-1 subjects with no sign of impaired glucose tolerance were randomized to oral insulin (7.5 mg per day)<sup>22</sup>. The original study failed to demonstrate that oral insulin delayed the clinical onset of T1D<sup>22</sup>. A post hoc analysis revealed a subgroup of individuals with high titer IAA who experienced a significant delay in clinical onset<sup>33</sup>. Recent follow-up of the subjects with high titre IAA who took oral insulin suggest that the preservation of  $\beta$ -cell function was maintained as long as the oral insulin was taken (Figure 3)<sup>34</sup>. TrialNet is currently recruiting participants to continue to test whether oral insulin is effective to prevent diabetes in relatives at risk for T1D (<http://www.diabetestrialnet.org/studies/oral-insulin.htm>).

#### *Nasal insulin*

Insulin has also been used nasally in secondary prevention trials in attempts to induce immune tolerance. In the Intranasal Insulin Trial (INIT), phase I and II trials, a double-blind, crossover design was used to study Australian IAA-positive subjects to first-degree relatives with T1D. INIT-I was completed 2004 with no significant effect on  $\beta$ -cell function but it showed some indications of immune tolerance to insulin<sup>35</sup>. INIT-II (NCT00336674) is an ongoing randomized; double-blind, placebo-controlled trial using nasal insulin (1.6 or 16 mg) and aims at assessing the effects of nasal insulin on islet autoimmunity. The Prediction and Prevention (DIPP) trial in Finland was a double-blind trial using nasal insulin in children with genetic risk and positive ICA and IAA<sup>36</sup>. In 224 children short acting insulin or placebo was administered intranasally once a day, but no protective effect was seen<sup>36</sup> nor did the nasal insulin modulate the characteristics of the IAA indicating that the insulin autoimmunity was already mature at the beginning of the intervention<sup>37</sup>. The importance of INIT and DIPP is the demonstration of safety and that ancillary or mechanistic studies demonstrated signs of immune tolerance to insulin. Future studies should build on this knowledge to include perhaps broader dose-response analyses and consider the possibility that the immune response to autoantigen may be closely related to the HLA-DQ genotype of the subject. In other words, insulin alone may not be sufficient when islet autoimmunity is spreading to IA-2A, GADA, or both.

#### *Glutamic acid decarboxylase (GAD65).*

GAD65 is a major autoantigen in T1D. While insulin autoimmunity is affecting the young, GAD65 autoimmunity is less sensitive to age. Alum-formulated recombinant human GAD65 tested in Phase II and III clinical trials were found to be safe<sup>38-40</sup>. The immunomodulating effect seemed to include the induction of Treg cells<sup>16, 38</sup> and the residual beta-cell function in newly diagnosed T1D patients. We have randomized a total of 50 children in the trial Diabetes Prevention – Immune Tolerance (DIAPREV-IT) to either placebo or alum-formulated GAD65 in a prime and boost design

(NCT01122446). All children have entered the study and results will be obtained in 2015. So far, there are no drug related adverse events. Investigation of the children at baseline (4 -18 years) revealed significant heterogeneity already before the first injection of the study substance (Figure 4). The data suggest that some children with GAD65 and at least one more islet autoantibody already show beta-cell function derangements. Eight children had reduced first phase insulin release (FPIR), five children had impaired glucose tolerance (IGT) after an oral glucose tolerance test (OGTT) and four children reduced ability to clear plasma glucose (Figure 4). However, only two children were abnormal in all three tests and four others shared at least two abnormalities. These data suggest that  $\beta$ -cell control of blood glucose vary markedly between children with at least two islet autoantibodies. This observation is important when secondary prevention trials are designed. It will be critical to define inclusion criteria to secure a homogeneous study population to increase the odds to detect effects on  $\beta$ -cell function by the intended treatment. Again, GAD65 alone may not be sufficient when islet autoimmunity is spreading to IA-2A, IAA, ZnT8A or all three. Ancillary or mechanistic studies, in particular of blood T, B and NK cells as well as monocytes may be important to further understand immune responses to islet autoantigens in the already islet autoantibody positive subject. A distinct weakness that may be possible to correct in future studies is that insulin has not been given with alum nor has oral or nasal GAD65 been tested.

In summary so far, the following are examples of secondary prevention trials currently in progress: the CD3 monoclonal antibody Teplizumab (NCT01030861), DIAPREV-IT (NCT01122446), INIT II (NCT00336674), DPT-1 Oral insulin (NCT00419562), and Intranasal Insulin for Prevention of T1DM (NCT00223613).

#### **4. Intervention – treating subjects who have been diagnosed with type 1 diabetes.**

Tertiary prevention or intervention trials recruit patients with newly diagnosed T1D. The aim of these trials is to preserve (or better increase) levels of c-peptide detected at the time of clinical diagnosis. A first intervention was reported in 1978 (ref) in three patients testing the effect of prednisone and azathioprine. After the demonstration a few years earlier of HLA association with T1D and demonstration of ICA, T1D began to be viewed as an autoimmune disease. Immunosuppression was therefore expected to be beneficial. The notion was born that newly diagnosed T1D patients should be treated with immunosuppression. Numerous open-label and small studies would follow. Essentially all immunosuppressive agents that would come on the market were to be tested on T1D patients<sup>41</sup>. None of these many drugs convincingly preserved the residual  $\beta$ -cell function present at the time of clinical diagnosis. Side effects and adverse events were common.

Intensive insulin regimen in patients with new-onset T1D was early proposed to preserve the remaining  $\beta$  cells and enhance their functionality<sup>42</sup>. Intensive insulin treatment is therefore used as the basic therapy for all newly diagnosed T1D patients who are randomized into intervention trials. Most investigators have a set goal for treatment to reach HbA1c levels that are as close to normal as possible. Regardless of treatment – non autoantigen- or autoantigen-specific insulin treatment will have to be taken into account. Autoantigen-specific immunomodulation have also been tested, so far with varying success to preserve residual  $\beta$ -cell function.

##### **a. Non-autoantigen intervention.**

Non-autoantigen specific drugs have been tested in several controlled and less controlled studies. A summary of already completed studies can be found on the PICCTURE (<http://www.ludc.med.lu.se/research-units/diabetes-and-celiac-disease/research-projects/diabetes-type-1-prediction-early-pathogenesis-and-prevention/piccture-activities/>) website and will therefore not be reviewed. Controlled clinical trials have been carried out with specific immunosuppressive agents including CD3 monoclonal antibodies. Anti-CD3 biologicals have shown promising results in smaller phase II trials. The following is a brief summary of recent or on-going trials and their rationale using non-autoantigen intervention.

#### *IL-2 receptor antibody (Zenapax).*

Daclizumab phase II trial, sponsored by Hoffmann-LaRoche and Facet Biotech, Prevention of Diabetes Progression Trial (PDPT) is testing the safety of Zenapax (NCT00198146). Zenapax (daclizumab) is an immunosuppressive, humanized IgG1 that binds specifically to the alpha subunit (p55 alpha, CD25, or Tac subunit) of the human high-affinity IL-2 receptor. The rationale is to inhibit T cells. The outcome will be of considerable interest as the TDGC GWAS study found that a genetic variant of the IL2R was associated with T1D in the <sup>3</sup>. Results from the trial are not yet available.

#### *CD3 monoclonal antibodies.*

Several clinical studies and trials have been carried out with CD3 monoclonal antibodies from different manufacturers (for a review see<sup>44</sup> ref). The reader is referred to extensive reviews of animal studies providing support for the rationale of using CD3 antibodies in intervention studies (ref).

Recently, two monoclonal CD3 antibodies have been extensively studied, oteelixizumab (ref) and teplizumab (ref).

The Phase II trial with oteelixizumab, a humanized non-mitogenic CD3 (ChAgly CD3) monoclonal antibody in newly diagnosed T1D patients suppressed the rise in insulin requirements over 48 months but the effect was related to age and residual c-peptide at diagnosis<sup>43</sup>. The subsequent industry-sponsored Phase III trial (Durable-Response Therapy Evaluation For Early or New-Onset Type 1 Diabetes – DEFEND, NCT00678886) used a cumulated dose of 3 mg as compared to 48 mg for the phase II study. As the end-point of this lower-dose study was not reached both DEFEND and DEFEND-2 was terminated. The four year follow-up of the patients in the Phase II clinical trial indicated a delayed the rise in insulin requirements of patients (43). Furthermore, in a subgroup of the Phase II trial patients it was shown that recall immunity was preserved adding to the aspect of safety (ref).

Treatment with teplizumab resulted in improved C-peptide responses and clinical parameters in T1D for at least 2 years in the absence of continued immunosuppressive medications (ref). The subsequent phase III clinical trial (NCT00385697) had modified end-points as the primary composite outcome was the percentage of patients with insulin use of less than 0.5 U/kg per day and glycated haemoglobin A(1c) (HbA(1C)) of less than 6.5% at 1 year. Although the composite end-point was not met it was noted after one year that all patients (100%) in the placebo group took insulin compared to 5% among the teplizumab-treated patients. Mechanistic studies suggest it is possible to monitor antigen-specific T cells after teplizumab treatment (ref), which should be encouraged in future clinical trials. It is a distinct drawback to progress that industry-conducted trials such as DEFEND-1 and -2 and PROTÉGÉ, have been discontinued and recruitment suspended leaving patient results and follow

up data unavailable for further analyses.

*Rituximab – an CD20 monoclonal antibody.*

TrialNet conducted this Phase II – III study (NCT00279305) in newly diagnosed T1D patients<sup>46</sup>. The data in Figure 5 demonstrate that  $\beta$ -cell function was preserved. The significant effects of this B lymphocyte monoclonal antibody was surprising to some as T1D is usually regarded as a T cell mediated disease. The results underscore the need for further studies on the role of B lymphocytes in maintaining the chronic islet autoimmunity. In mice, it has been demonstrated that there is a significant cross-presentation of autoantigens to CD8+ T cells that precipitates diabetes (ref). Rituximab proved effective in blocking the immune response to neoantigens (ref) but did not affect already established autoantibodies such as GADA and IA-2A (ref) while insulin and recall antigen antibodies were reduced<sup>47</sup>. The observations on autoantibody and neoantigen responses is underscoring the importance of the B cell as an antigen-presenting cell that may not only interact with T helper cells but also directly with other antigen presenting cells such as dendritic cells to induce autoantibody formation (Figure 6). However, as the safety of Rituximab is of major concern, the future use of this monoclonal antibody in intervention studies requires thoughtful considerations. Other approaches that would target the APC-B cell or the B-T cell synapses will be of considerable interest as it is still unclear whether inhibiting islet autoantibody formation will be associated with preserved  $\beta$ -cell function (Figure 6).

*IL-1 $\beta$  receptor antagonist and IL-1 $\beta$  antibodies.*

IL-1 $\beta$  has long been considered a beta-cytotoxic cytokine. Anakinra, an IL-1 $\beta$  receptor antagonist, was therefore used to treat 15 children within one week of diagnosis with daily for 28 days and then followed for 6 months (NCT00645840). It was concluded that Anakinra was well tolerated but that the drug did not preserve  $\beta$ -cell function<sup>48</sup>. The future use of Anakinra in intervention trials is unclear. The Anti-Interleukin-1 in Diabetes Action (AIDA) trial is using anakinra (NCT00711503) as well as the TrialNet study with canakinumab, which is a human interleukin-1 $\beta$  (anti-IL-1 $\beta$ ) IgG1 monoclonal antibody (NCT00947427). The possible use of Anakinra in combination trials may be considered dependent on its safety profile in children and young adults.

*BCG vaccine*

The rationale to test BCG vaccination is based on animal studies. A number of BCG studies have been completed showing some (ref) or no (ref,ref) preservation of c-peptide. A recent registered human trial is focused on establishing a possible reduction in self-reactive T lymphocytes (NCT00607230).. The study has stopped recruiting and would seem to represent an exploratory description on BCG vaccination compared to saline in T1D patients. It is unclear how the information will be used for possible future studies on non-autoantigen specific immunomodulation.

*ATG*

Anti-thymocyte globulin (ATG) was found to induce short-term benefits in the NOD mouse primarily through inducing immunoregulation rather than depletion of T cells. In humans ATG is thought to decrease insulin requirement in patients with new-onset T1D, however serious adverse effects such as transient thrombocytopenia are major

drawbacks. In the START (Study of Thymoglobulin to Arrest Newly Diagnosed Type 1 Diabetes) phase II study (NCT00515099), Thymoglobulin® are administered daily to newly diagnosed (older than 12 years of age) T1D patients in escalating doses over 4 days. The end-point of the START trial, supported by the Immune Tolerance Network is a mixed meal tolerance test for c-peptide after 12 months of diabetes duration. The second clinical trial (NCT00190502) is carried out in the Czech Republic with a similar design. The progress in this placebo-controlled clinical trial is unclear.

*Abatacept* TrialNet initiated this study with the rationale to determine whether treatment with CTLA4-Ig (Abatacept) in newly diagnosed T1D patients would preserve mixed meal tolerance test stimulated C-peptide compared to placebo (NCT00505375). CTLA-4 is thought to be involved in modulating immune responses through inducing co-stimulatory signals, which are important for T lymphocyte activation. CTLA4 immunoglobulin (CTLA4-Ig) is proposed to regulate, but not delete, T lymphocytes through inhibiting their stimulatory pathway of activation, therefore is considered relatively safer than other immunosuppressive agents. The first report of this phase II clinical trial demonstrated that c-peptide AUC was significantly higher at 2 years with abatacept than with placebo<sup>49</sup>. It was concluded that T-cell activation still occurs around the time of clinical diagnosis of T1D but that the beneficial effect was parallel to that of the placebo after the initial preservation of c-peptide. The safety needs to be reviewed in detail before proposing yet other interventions with this immunomodulating compound.

#### *DiaPep277*

DiaPep277 is a peptide related to heat shock protein and has immunomodulatory characteristics. The mechanisms of action are not fully clarified. Treatment with DiaPep277 has been safe. Two phase-III clinical trials are in progress (NCT01103284 and NCT00615264). Efficacy phase III clinical trials of DiaPep277 are underway in newly diagnosed T1D patients (DIA-AID) as well as in newly diagnosed T1D adults (DIA-AID2) to test whether DiaPep277 preserve residual c-peptide. Meal-stimulated c-peptide will be tested during two years of follow-up after 10 injections of DiaPep277 or placebo. Additional clinic trials are in the planning<sup>50</sup>.

b. Autoantigen specific intervention.

#### *Insulin peptides*

A Study to Evaluate NBI-6024 in Adult and Adolescent Patients With New Onset of Type 1 Diabetes Mellitus (NCT00873561) was a phase I clinical trial with the altered peptide ligand of insulin<sup>51</sup>. NBI-6024 is an insulin B chain:9-23 vaccine and it was able to shift the interferon-gamma-producing T helper (Th1) lymphocyte into Th2 regulatory T cells. However, treatment with NBI-6024 at repeated doses of 0.1, 0.5, or 1.0 mg did not improve or maintain  $\beta$ -cell function.

Safety and functional responses to a proinsulin peptide has also been reported (ref) and further studies on the approach to modulate the autoimmune response to proinsulin and insulin are needed. Such studies should take into account differences in the way peptides are presented on relevant HLA-DR and -DQ molecules. It may be important in this regard to further investigate the uptake and processing of proinsulin and insulin by B lymphocytes expressing insulin-specific BCR.

#### *Proinsulin DNA vaccine*

BHT-3021, a plasmid encoding proinsulin was designed to tolerize the immune system to proinsulin (NCT00453375). The safety of this drug was tested in a randomized, blinded, placebo-controlled multi-center study<sup>52</sup>. The rationale is to test whether expression of this plasmid in vivo would produce proinsulin able to turn off autoimmunity. The study has been completed but results are yet to be reported.

#### *GAD-alum*

GAD-alum tested in Phase II clinical trials with some effect on preserving residual c-peptide<sup>39, 40</sup> was tested in an additional phase II trial with a dose-regiment that differed from previous trials<sup>53</sup>. Two or three doses of subcutaneous GAD-alum across 4-12 weeks did not alter the c-peptide disappearance rate during 12 month in newly diagnosed T1D patients (NCT00529399). Similar failure to affect the c-peptide disappearance rate over 15 months follow-up was found in a phase III study (NCT00723411) carried out in Europe<sup>54</sup>. The lack of effects of the GAD65-alum approach has complicated further clinical investigations especially as the possible effects of the treatment on immune parameters such as the induction of Treg cells<sup>38, 55</sup> are still to be reported.

#### **d. Combination interventions**

It has been argued that monotherapy in newly diagnosed T1D will be insufficient as the islet autoimmunity has been established against several autoantigens years before the clinical onset of diabetes. It has also been argued that the transient effects of immunosuppression to preserve residual c-peptide may be overcome by the use of combination therapy rather than monotherapy. The use of combination therapies with two agents tested individually and together against two placebo injections also offer statistical advantages in addition to the possibility that the interaction between two drugs may be possible to delineate. Effects of a combination therapy design has been reported in two studies.

#### *IL-2 (proleukin) and Sirolimus (Rapamycin)*

The Immune Tolerance Network (ITN) combined IL-2 (Proleukin) and Sirolimus (Rapamycin) in a phase I trial (NCT00525889).. The rationale was that this drug combination was found to be effective for long-term diabetes prevention in the NOD mouse. The study is open-label, uncontrolled safety trial. Participants are adults, 18-45 years old who were diagnosed with T1D within 4 years. It was reported that regulatory T cells (Tregs) increased within the first month of therapy, yet clinical and metabolic data demonstrated a transient worsening in all subjects. The authors conclude that their results highlight the difficulties in translating therapies to the clinic (ref).

#### *MMF and Daclizumab*

A multi-center, three-arm, randomized, double-masked, placebo-controlled clinical trial carried out by TrialNet. Mycophenolate Mofetil (MMF/CellCept) and the anti-IL2 receptor monoclonal antibody, Daclizumab (DZB/Zenapax) was tested in patients 8-45 years of age diagnosed within 3 months (NCT00100178).. This trial was to assess whether this combination would preserve the residual  $\beta$ -cell function<sup>56</sup>. However, neither MMF alone nor MMF in combination with DZB had an effect on the loss of c-peptide<sup>56</sup>. Adverse events were common and the safety of this



combination therapy needs careful consideration especially events that included EBV activation.

Other combinations may be worth considering for future intervention trials. Combining the BCG vaccine with an autoantigen may enhance the effect of immunomodulation with BCG. Previous intervention trials should be revisited to analyze to what extent reported c-peptide preservation was related to IAA levels at entry and the amount of insulin used at the time when BCG was given. Other questions related to combination therapy relates to the use of immunosuppressive monoclonal antibodies which may be better tolerated if give together with substances that are considered immunomodulating such as vitamin D or omega-3 fatty acids.

#### **e. Future directions**

The summary of past and on-going clinical trials in islet autoantibody positive subjects (prevention) or in the newly diagnosed T1D patients (intervention) show little promise to achieve the major end-point of preserving  $\beta$ -cell function. The trials, be it non-autoantigen or autoantigen-specific have represented a therapy with only one agent. The rationale and design has often been based on animal studies. In retrospect, so-called preclinical studies in mice and rats may be useful but sometimes misleading despite providing a strong rationale for clinical trials in humans. This is not surprising since the NOD mouse and the BB rat, the two prime rodents developing spontaneous autoimmune diabetes have an etiology and pathogenesis different from the human condition. This fact still does not disqualify studies of spontaneously diabetic animals as they provide mechanistic clarification that is often not possible to achieve in human studies. Future trials to prevent either islet autoimmunity (primary prevention) or T1D in islet autoantibody-positive subjects (secondary prevention) may however need alternative approaches based on what is known in humans as the spontaneously diabetic NOD and BB rats do not present comparable etiology and pathogenesis of the very early stages of the disease. Preclinical studies may still have to focus on toxicity and safety.

It is important to note that the rapid progress in developing humanized monoclonal antibodies against a variety of immunological targets are often based on the need for more specific immunosuppression in transplantation or in severe immune-associated disorders. The safety profile of these biologicals needs careful review before tested in T1D prevention or intervention trials.

None of the many immunosuppressive agents tested so far have preserved  $\beta$ -cell function long-term. At best, there have been transient effects but further studies have had to be abandoned due to safety issues or severe side effects of the immunosuppressive drug (ref,ref). Safety remains a major concern in clinical trials with immunosuppressive agents. The risk benefit analysis always need to take into account the fact that it is possible to live an almost normal life with daily insulin injection despite all restrictions imposed on a person with T1D. Whether a future trial is prevention or intervention safety and long-term effects will have to be a major concern. So far, the safety profile in trials with insulin<sup>22, 36</sup> and GAD65<sup>38, 40</sup> autoantigen immunomodulation has been high.

The different approaches of autoantigen administration have revealed effects on secondary end-point measures such as increased numbers of Treg cells. Although  $\beta$ -cell function has not been fully preserved<sup>40</sup> and reproducible<sup>53, 54</sup> these high safety studies makes it possible to design trials to evaluate dose and exposure dependent parameters. Recent advances in flow cytometry, immunogenetics in combination with

$\beta$ -cell function tests such as the MMTT should make it possible to dissect the impact of autoantigen immunomodulation on the immune response in relation to  $\beta$ -cell function. In the wake of first-in-man studies of IA-2 and ZnT8, combination therapy with for example proinsulin and GAD65, alone and in combination (Table 1) should make it possible to evaluate the immune response to this autoantigens particular in secondary prevention trials. Dose-dependent analyses in addition to studies of the route of administration will also be critical to our understanding of safe immunomodulation.

Immunomodulation with autoantigen possibly combined with immunosuppressive therapy has been much debated in relation to rodent studies<sup>57</sup>. Based on the recent failures it may be rightly questioned whether immunomodulation – be it prevention or intervention- with a single autoantigen will ever be sufficient. Provided the treatment is safe, it cannot be excluded that the simultaneous administration of GAD65, IA-2, ZnT8 and proinsulin may be more efficacious than either autoantigen alone (Table1). The route of administration needs further exploration also in intervention trials. Oral insulin was tested but what about oral GAD65 or any of the other autoantigens? Shouldn't they also be given orally? Similarly, should alum-GAD65 be tested together with alum-formulated proinsulin? Or with any of the other autoantigens formulated the same way?

Our understanding of the function of the human immune system lags behind that of the mouse but current human immunology studies are slowly diminishing the gap. Safe immune tolerance trials may provide a novel approach to dissect the mechanisms by which the human immune system responds to immune therapy with autoantigens. It is important in this regard that attention is paid to the HLA genotype of the subjects. There is a need to further explore the role of HLA-DQ types when clinical trials are designed. Although CD4+ T cells expressing TCR that recognize autoantigen-peptides presented on HLA-DR or -DQ heterodimers remain in undisputable target for prevention, it seems equally important that B cells expressing BCR reactive an islet autoantigen are targeted (Figure 6). As shown in ex vivo experiments, B cells are effective antigen presenting cells<sup>58, 59</sup>. Recent studies in the mouse provide evidence that there is a so-called cross-talk between B lymphocytes and CD8+effector T cells that may lead to diabetes (ref). The chronic autoimmune response in subjects with islet autoantibodies may be best treated to eradicate the B cells or plasma cells that continue to produce islet autoantibodies. Targeting B cells expressing BCR recognizing an islet autoantigen may also be an effective secondary prevention therapy. In terms of autoantigen-presentation, controlled clinical trials offer a unique opportunity to dissect the relationship between HLA-DQ genotypes and immune responses to autoantigens alone, or in combination. Such studies may also help to design novel drugs that interfere with the binding and presentation of autoantigen peptides on HLA-DQ heterodimers. In this regard, studies in patients with Latent Autoimmune Diabetes in the Adult (LADA) may be particularly important. Newly diagnosed T1D patients older than 18 but younger than 35 years of age and LADA patients above 35 years of age are expected to outnumber T1D patients younger than 18 years of age at least by a factor of three, at least in Sweden<sup>60</sup>. Interventions studies will not take long to fill and subjects may be matched for HLA-DQ genotypes. The subjects already have diabetes and will be able to consent to blood sampling volumes unattainable in children but needed for more extensive immunological assays. Although c-peptide may not be as sensitive an outcome as in studies of children this may be countered by selecting participants with similar base-line levels<sup>60</sup>.

TrialNet and the Immune Tolerance Network are international networks supported by the National Institutes of Health (NIH). Both networks have established an infrastructure for trials for predicting and preventing T1D. Within these networks, relatives of T1D patients are screened for disease risk and then either followed for the disease natural history or, if possible randomized into clinical trials. There seem to be little reason not to include subjects at high HLA-DQ risk for T1D in the screening effort. This important as only about 13-15% of newly diagnosed T1D patients have a first-degree relative with the disease. Current screening technology for both HLA-DQ typing and islet autoantibody analyses have sufficiently high capacity also to include the general population<sup>20</sup>. Adding general population children and young adults to the screening effort will identify individuals with multiple autoantibodies to be eligible for secondary prevention studies. Investigator-initiated clinical trials with or without a cooperation with networks such as TrialNet and the Immune Tolerance Network are needed to carry out smaller and more efficient studies. Such trials need to have elements of dose-escalation, route of administration and more immunological outcomes than used to date. Drugs used in combination often increase statistical power and study groups may be kept smaller. Trials may take less time. The traditional focus on T cells may be combined with B cells as the target especially when islet autoantibodies are used as the read-out of islet autoimmunity. We need to obtain therapy that is able to reduce islet autoantibodies to test the hypothesis that the presence of islet autoantibodies reflect an on-going, chronic autoimmune disease directed against the pancreatic  $\beta$  cells. It cannot be excluded that this goal can be reached by combining immunosuppressive therapy with CD3 monoclonal antibodies with exposure to any or all of the four autoantigens to increase the chances of inducing immunological tolerance.

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*Text box*

Textbox 1. Islet autoimmune markers used to randomize subjects in clinical trials of prevention and intervention.

HLA-DQ haplotypes	HLA-DQ A1*03:01-B1*03:02 HLA-DQ A1*05:01-B1*02:01	
HLA-DQ genotypes	Very high risk: A1*05:01-B1*02:01/ A1*03:01-B1*03:02 High risk: A1*03:01-B1*03:02/A1*03:01-B1*03:02 A1*03:01-B1*03:02/A1*01:01-B1*05:01 A1*03:01-B1*03:02/A1*01:02-B1*06:04 A1*03:01-B1*03:02/A1*04:01-B1*04:02 A1*05:01-B1*02:01/ A1*05:01-B1*02:01 A1*05:01-B1*02:01/ A1*01:02-B1*06:04 A1*03:01-B1*03:02/A1*03:01-B1*03:03 <b>Total genotype frequency</b>	% in T1D: 28 11 9 5 5 5 4 3 <b>70</b>
Islet autoantibodies	IA-2A GAD65 ZnT8R ZnT8W Insulin ZnT8Q  <b>One or several islet autoantibodies</b>	73 56 54 47 33 32  <b>93</b>

Data from the Swedish Better Diabetes Diagnosis (BDD) study representing HLA-DQ genotyping and islet autoantibody analysis from more than 3,000 patients diagnosed with T1D during May 2005 – August 2010 <sup>61</sup>. ZnT8 autoantibodies were against the arginine (R), tryptophan (W) or glutamine (Q) at position 325 in the translated protein.



Table 1. Factorial design of a combination therapy approach to type 1 diabetes prevention and intervention trials.

Group		
I	Placebo	Placebo
II	Compound A	Placebo
III	Placebo	Compound B
IV	Compound A	Compound B

## LEGENDS TO THE FIGURES

**Figure 1.** Representation of the type 1 diabetes etiology and pathogenesis. The genetic risk is conferred by HLA on chromosome 6. Two haplotypes DQA1\*03:01-B1\*03:02 (abbreviated DQ8) and DQ A1\*05:01-B1\*02:01 (abbreviated DQ2) are the two major risk determining factors.

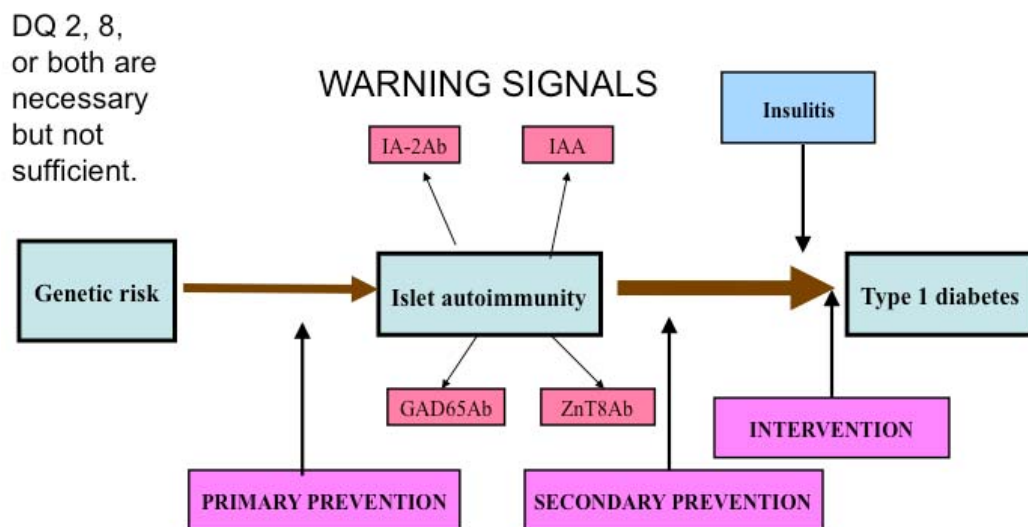
**Figure 2.** The number of islet autoantibodies determine the rate of progression to the clinical onset of type 1 diabetes.

**Figure 3.** Follow-up for 9 years for patients participating in the DPT-1 oral insulin trial. The data suggest that oral insulin was efficacious as long as the drug was not discontinued.

**Figure 4.** Baseline beta-cell function test in 21/50 children subsequently randomized to placebo or treatment with alum-formulated GAD65.

**Figure 5.** Rituximab, a monoclonal antibody against CD20 specifically expressed on B lymphocytes preserved residual c-peptide after mixed meal tolerance tests.

**Figure 6.** Cartoon of the synapses between antigen presenting cells and B cells as well as between B cells and T cells. Understanding these cellular interactions may be critical to the development of islet autoimmunity.

**Figure 1.**

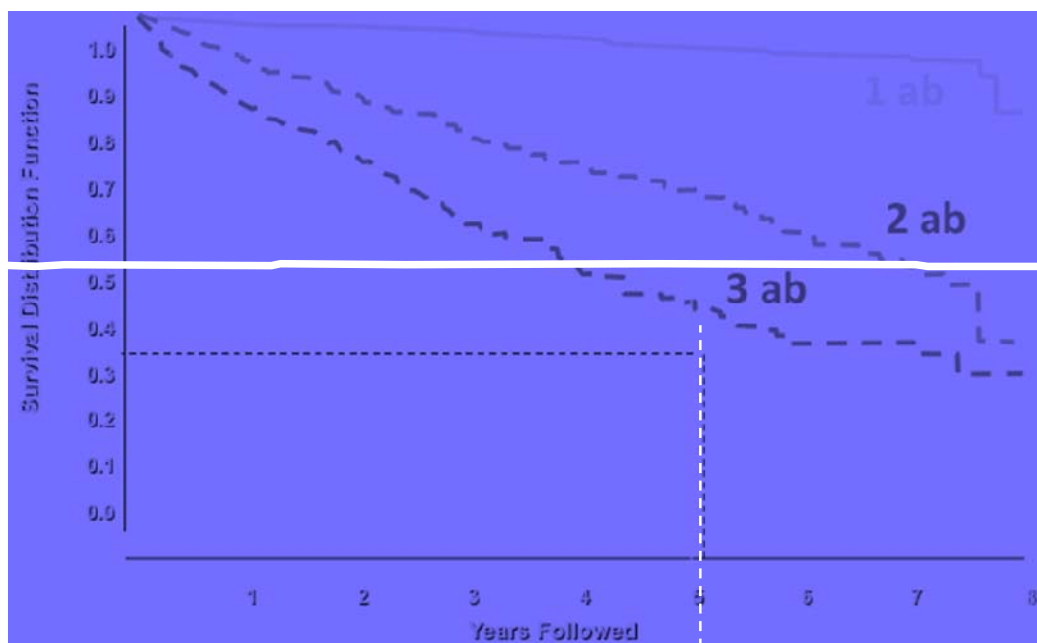
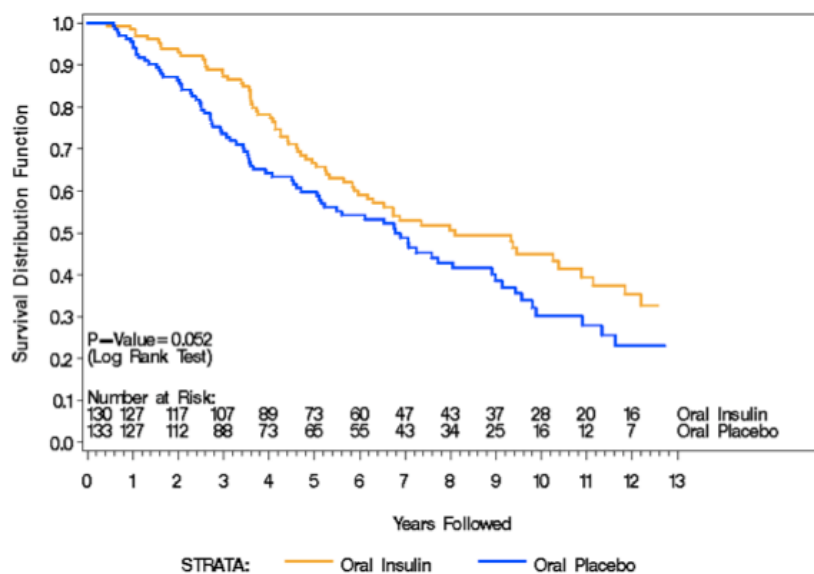
**Figure 2.**

Figure 3.

**Overall DPT-1 Follow-up Study - Time to Diabetes - By Treatment**  
**Subset: IAA Confirmed  $\geq 80$  nU/ml – median follow-up 9.1 yrs**



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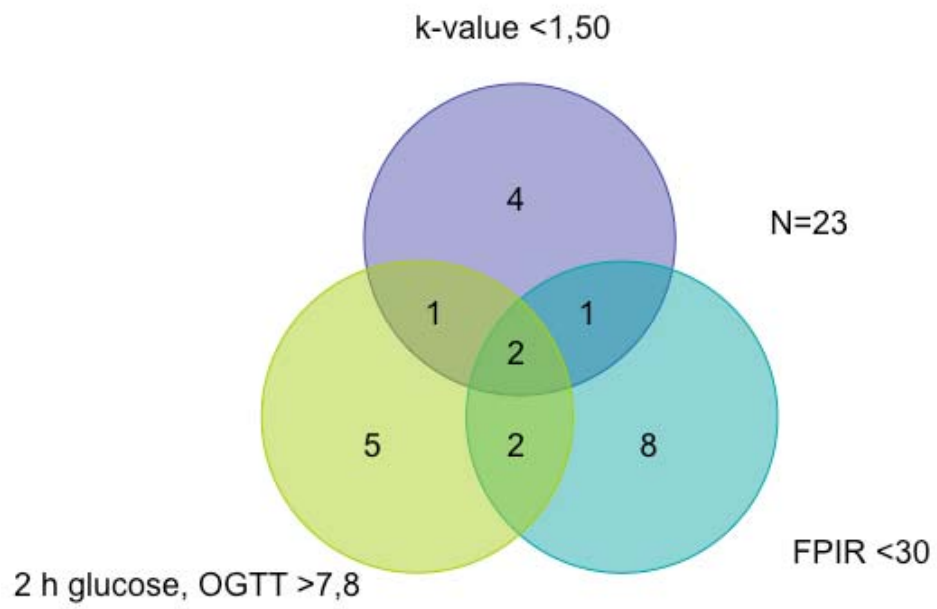
**Figure 4.**

Figure 5.

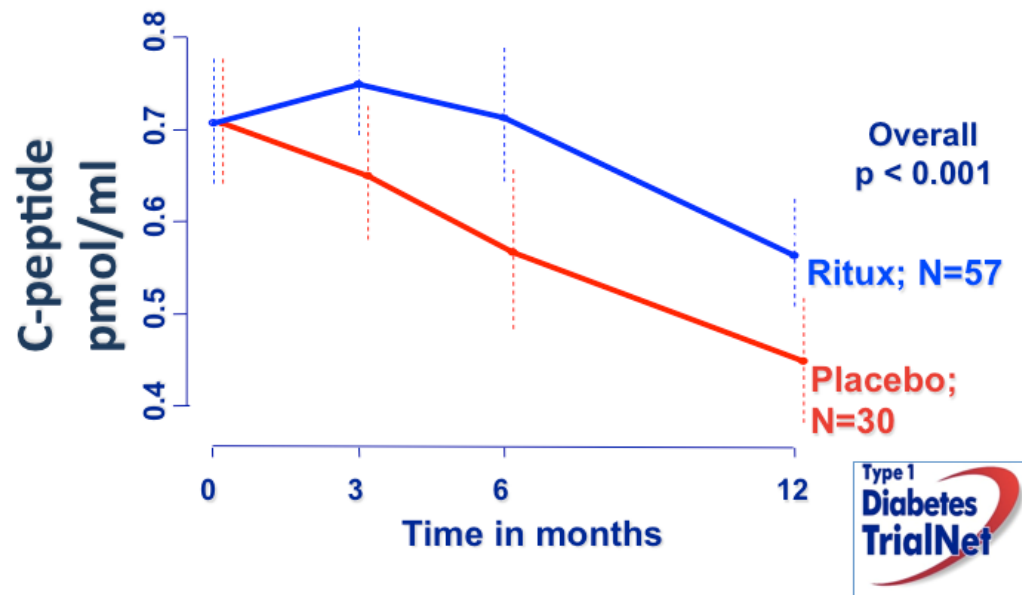


Figure 6

