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Prediction and prevention of type 1 diabetes in children

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Abstract. Type 1 diabetes (T1D) is a chronic T-cell mediated autoimmune disease characterized by destruction of beta cells. Although new data have better defined the complex etiology underling the interrelation of genetic and environmental factors in the natural history of T1D, relevant pieces of the puzzle still are missing. Genetic predisposition is mainly associated to some histocompatibility leukocyte antigen (HLA) alleles; however, recent data suggest that new as well as still unknown genes might better define the complex multigenetic risk of the disease. In addition to the genetic effects, the concordance in familial aggregation in T1D indicates a pivotal role of environmental factors in the course of the disease, facilitating autoantibodies production. JDRF has recently proposed a new early stage of T1D according to which the detection of two or more autoantibodies in the blood, might describe those children at increased risk of developing T1D during the following years. In contrast to the improvements reached by prediction models, to date primary, secondary and tertiary prevention have still failed to achieve a safe and efficacious intervention strategies. Anyway, the most recent progresses in this field pave the way for future studies, with the aim of preventing T1D in children.

Key words: type 1 diabetes, prediction, prevention, children

Introduction

Type 1 diabetes (T1D) is a chronic T-cell mediated autoimmune disease characterized by immune-mediated destruction of pancreatic beta cells. In these complex events, genetic and environmental factors have been shown to represent the key modulators of the natural course of the disease. Genetic studies have explained the main role of the major histocompatibility

complex (MHC) genes, which encode for human leukocyte antigens (HLA) located on chromosome 6p21. However, genome analysis has also added a role of non-HLA genes by explaining a further 7–10% genetic risk (1). Environmental factors including viruses (in particular enteroviruses), food antigens (cow's milk proteins, nitrites, n-3 fatty acids) and toxins play an important role in genetically predisposed subjects, especially in the early stages of life. However, their role is not yet completely clarified (2). The loss of pancreatic beta cells leads to a progressive reduction of insulin production and increase of blood glucose and consequently to insulin administration and risk of short and long-term complications. According to the Juvenile Diabetes Research Foundation [JDRF], once autoimmunity has been developed, the progression to clinically evident T1D can be classified into three major
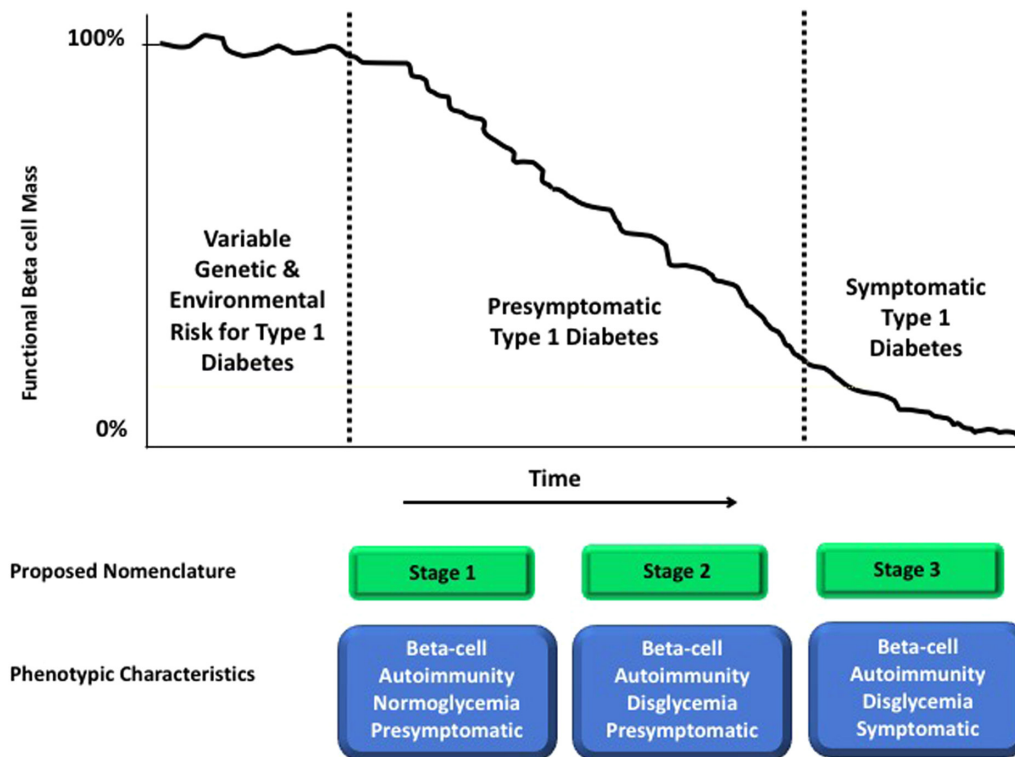
Fig. 1. T1D stages (proposed by the Juvenile Diabetes Research Foundation [JDRF] - Stage 1: beta-cell autoimmunity, normal blood glucose, no symptoms, Stage 2: beta-cell autoimmunity, impaired blood sugar, no symptoms, Stage 3: beta-cell autoimmunity, impaired blood sugar, presence of symptoms (3).

stages: 1) asymptomatic beta-cell autoimmunity with normal glycaemia, 2) asymptomatic beta-cell autoimmunity with hyperglycaemia and 3) symptomatic T1D (Fig. 1) (3). Although, the sequence of events that progressively leads to the activation of the production of autoantibodies has become predictable, the duration of each phase can widely differ among subjects. Today, the knowledge on genetic predisposition allows to stratify the risk of T1D. However, different genes and environmental 'triggers' can strongly influence the chronology of the appearance of different autoantibodies to different beta-cell antigens. As well, the number of autoantibodies has a significant influence on the natural history of the disease.

This article aims to provide an update about the ability to predict the onset of the disease and to describe the state of the art and future perspectives on the possibility of preventing the disease in children with different risk factors including: family history for T1D and genetic factors (HLA and other genes, other autoimmune diseases), immunological (number and titer of autoantibodies against beta cell antigens, markers of cellular immunity) and metabolic factors (reduced insulin production after intravenous or oral glucose load, hemoglobin A1c, fasting or postprandial hyperglycaemia).

Prediction

In recent years, many studies have been performed on the predictive value of the risk of developing T1D. These studies have been mainly focused on the role of family history for T1D, of the genes (allelic and haplotype variants of the HLA region, non-HLA genes) and of the immunological and metabolic biomarkers. The complete characterization of the effects of these

components might strongly affect the prediction of this complex multifactorial disease.
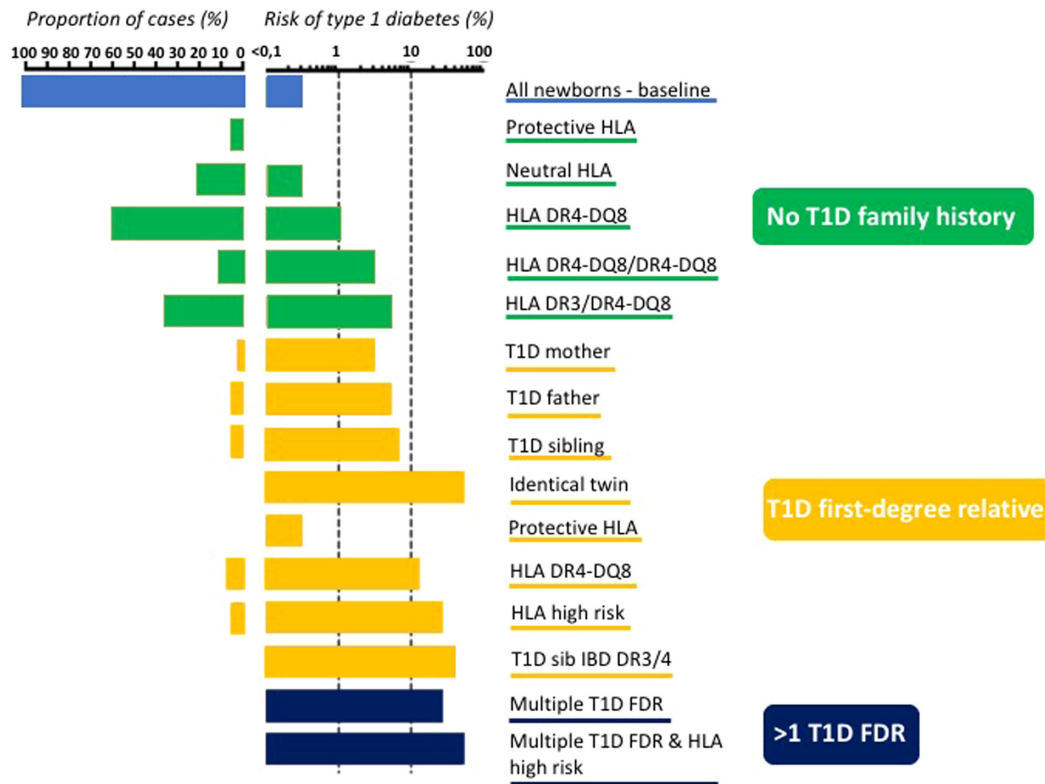
Family history

The familial aggregation of T1D, such as other autoimmune diseases, has been confirmed in several studies. The risk of developing T1D varies from 1 to 15% in siblings, parents and children of individuals with T1D, while the risk of developing the disease in the general population ranges from 0.12 to 0.4%. In Finland it has been shown that about 22% of newly diagnosed T1D children has a first- or second-degree relative with T1D (4). However, a negative family history is also reported in approximately 85% of cases with newly diagnosed T1D. The risk of developing T1D is markedly increased in children with affected first-degree relatives, ranging from about 6% in children (especially if the father is the parent with T1D), 5% in the siblings and between 23 and 50% in monozygotic twins (5). A monozygotic twin of a patient with T1D has a higher risk of developing the disease compared to a dizygotic twin; moreover, the expression of beta-cell autoimmunity does not differ between a dizygotic twin and the siblings of a child with T1D (5). This risk is also modulated by additional or protective risk factors, such as some HLA haplotypes or if more than one of the first degree relative is affected (Fig. 2) (5).

Genetic predisposition

HLA genes: Major histocompatibility complex genes (MHC) are located on genetic segment (~ 3.5 mega base) of chromosome 6 (6p21). The main MHC genes encode for human leukocyte antigens (HLA) that play an important role in the antigen presentation to T lymphocytes. Some HLA genes combined with the insufficient function of the regulatory T lymphocytes (TREG) induce autoimmune diseases. HLA-DR3-DQ2 and HLA-DR4-DQ8 haplotypes are strongly associated with T1D in Caucasian populations. In countries with high T1D incidence (such as Scandinavian countries), about 90% of children

with T1D has one or more of these haplotypes (6). For this reason, neonatal screening was used to identify children with increased genetic risk, in order to activate a follow up since birth with the aim of characterizing the appearance of autoantibodies against beta cells, lifelong. Polymorphisms of DRB1, DQA1 and DQB1 alleles have also shown to determine the genetic susceptibility, with a different susceptibility related to the haplotypes detected in different populations (6). Approaches of molecular biology provided further information on the role of HLA haplotypes in T1D pathogenesis. In fact, the location of aspartic acid at position 57 in the beta chain of DQB1 has a protective effect (DQB1* 06:02), while the presence of alanine is characteristic of haplotypes associated with an increased risk of developing T1D (genes DQB1* 02 and DQB1* 03: 02) (7). Furthermore, a role on the variation and association of different haplotypes in the alpha chain has been also demonstrated particularly for the association DQA1* 03-DQB1* 03:02 (7). Other loci in the HLA region may influence the genetic susceptibility to the disease such as the HLA class II, HLA-DP locus and some class I alleles (such as A* 24, B* 18 and B* 39). The characterization of HLA haplotypes therefore might be adopted to define a risk score for the appearance of autoantibodies against the beta cell. In fact, the anti-insulin antibodies (IAA) are the first detected in children with HLA-DR3-DQ2/HLA-DR4-DQ8, while anti-GAD antibodies in children homozygous for HLA-DR3-DQ2 (1–7). Similarly, other class II HLAs (HLA-DRB3, HLA-DRB4, HLA-DRB5) may also be associated with the production of autoantibodies and the risk of T1D. Therefore, the complete characterization of both the genetic components and the family history and particularly the combination of them need to be identified in order to properly assess the risk of developing T1D (Fig. 2) (5).

Non-HLA genes: Non-HLA genes are important but not sufficient to develop T1D, thus clearly suggesting a polygenic inheritance in most cases. Among the non-HLA genes

Fig. 2. Integration model characterizing the risk of developing T1D according to family history and Human Leukocyte Antigens compared to all newborn Caucasian infants (basic risk is 0.3–0.4%). The figure shows the stratified risk of developing T1D during the 20 years of life in different groups of subjects classified according to HLA risk as well as to the negative and positive family history. FDR: First Degree Relative (5).

associated with an increased risk of T1D, the most relevant are: insulin (INS), protein tyrosine phosphatase-non-receptor type 22 (PTPN22), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), IL-2 receptor (CD25), lectin-like ERBB3 gene and a locus located on chromosome 12q. However, genome-wide association studies (GWAS) have recently identified at least more than 60 other susceptibility loci (including UBASH3A, IL21, TNFAIP3, GLIS3IL2RA, NRP1, IL27) (8). These studies have shown both an increased risk and a protective role of different genes in the development of the disease (1–8). However, available data clearly show that the effects of these genes do not significantly affects the predictive value of the risk of developing T1D. In fact, by adding non-HLA genes in a screening

analysis, it is possible to identify the 83% (just 8% more) of the individuals who will develop T1D in contrast to the 75% obtained by the analysis of only HLA genes (1). Further studies could identify new and probably more relevant non-HLA genes associated with the risk of developing T1D.

Cellular immunity

During the last few years the knowledge about the role of cellular immunity in beta cell destruction has significantly improved. After birth an immunological balance is established in which self-reactive T-cells against beta cells are controlled by the regulatory T cells (TREG). In genetically predisposed subjects, if this state of immunotolerance is threatened
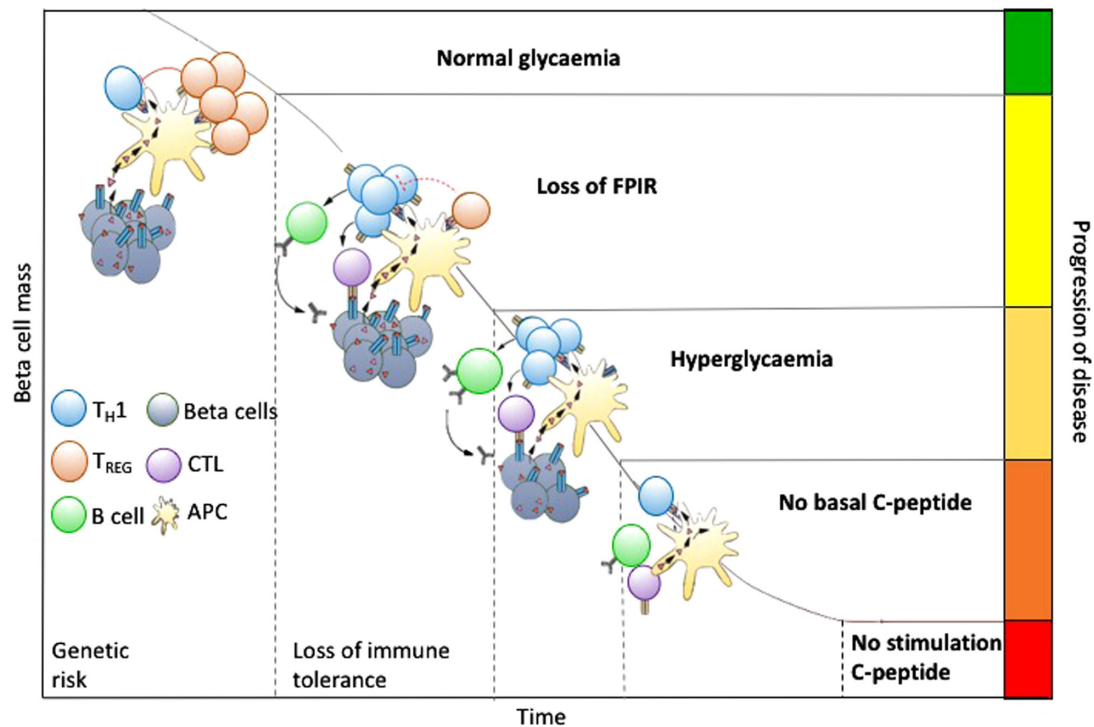
Fig. 3. Progression of T1D according to immune- and pathogenic mechanisms. After birth an immunological balance is established in all children. In details, the regulatory T cells (T_{REG}) control self-reactive T-cells reactivity against beta cells. If this state of immunotolerance is threatened by some factors (still unidentified, probably including viruses, some food antigens, toxins, etc.), T_{REG} are no longer able to control autoreactive lymphocytes, with consequent activation of B lymphocytes and production of autoantibodies against beta cells and also activation of cytotoxic lymphocytes (CTL). Loss of First Phase Insulin Release (FPIR) corresponds to the progressive decline of beta cell mass and less effective response of beta cells to increased blood sugar. In this phase the T_{REG} attempting to preserve beta cells inhibiting autoreactive lymphocytes delay clinical manifestation of T1D. After the onset of diabetes the beta cells are destroyed and the child becomes totally insulin dependent. APC, antigen presenting cells; CTL, cytotoxic T lymphocytes; T_{H1}, T helper 1; T_{REG}, T regulatory lymphocytes; B cell, B lymphocytes; the autoantigens of the beta cells are indicated by the triangles (9).

by some factors (mostly still not identified but probably including viruses, toxins and some food antigens), the TREGs are no longer able to control the self-reactive T lymphocytes. Thus, this state determines the activation of B lymphocytes (which produce antibodies against beta cell antigens) and of cytotoxic lymphocytes (which attack beta cells and induce apoptosis) (Fig. 3) (9). Inflamed pancreatic islets (in patients with T1D or in pancreas transplants from a healthy donor to their twin with T1D), are massively infiltrated by CD8+ T lymphocytes (9). The autoreactive cells specific for pancreatic

islets are observed in patients with T1D, but are also detectable in the peripheral circulation of people without T1D or without any other autoimmune disease. As certainly well known, there are many T lymphocyte populations, whose activation and proliferation are finely regulated. In particular, the expansion of one population negatively regulates the generation and expansion of another (for example, there is a cross-regulation between T helper 1 [TH1], TH2 and TH17 CD4+). The interactions between this subset of lymphocytes determine the progressive beta cells destruction and clinically manifest

T1D (Fig. 3) (9). There are two main groups of regulatory T lymphocytes, classified on the basis of the expression of the forkhead box P3 (FOXP3) transcription factor (9). FOXP3$^+$ TREG cells play a key role in maintaining immunological tolerance through the suppression of self-reactive T lymphocytes (9). FOXP3$^−$ CD4 cells also have regulatory activity (for example, Tr1 cells produce IL10 after stimulation with their target antigen). Some studies have shown that the strengthening of these regulatory T cells could be very important in the prevention of T1D (9). Other studies on mechanisms of cellular immunity could improve the ability to counteract the activity of some lymphocyte subset; for example, the use of a monoclonal antibody against CD20 lymphocytes (rituximab) has been shown to be effective in preserving beta cell function in patients with newly diagnosed T1D (10). However, further studies are needed in order to completely define the complex role of regulatory T cells in the natural history of the disease.

Humoral immunity

Ongoing research is focused on the identification of auto antigenic targets of the autoimmune process; preliminary studies have shown that the production of one or more autoantibodies against beta cells results in a significant increasing risk of developing T1D (11). The chronic course of the disease ranging from the unbalanced immune regulation to autoantibodies production and diabetes onset related to beta cell depletion is primarily characterized by the development of a state commonly defined as the "epitope spreading" (10). Particularly, during this phase a higher number of islet autoantigens reacts with T cells and autoantibodies affecting the progression of the disease (10). The autoantibodies currently used for the screening of T1D are those against the main beta cell antigens including: glutamate decarboxylase (GAD), Islet Antigen 2 (IA-2), insulin (IAA), zinc transporter 8 (ZnT8); while the old Islet Cell Antibodies (ICA) are not currently used (10–12). Other autoantigens have been described including: ICA69 kDa (ICA69), the 'islet-specific glucose-6-phosphatase catalytic subunit-related protein' (IGRP), the chromogranin A (ChgA), the insulin receptor, some heat shock proteins, the antigens jun-B-16, CD38, peripheral and glial fibrillary acidic protein (GFAP). Although, most of them are well characterized in subjects with newly diagnosed T1D, their use in the clinical practice is poorly relevant (10–13). Relevant studies have clearly showed that the co-occurrence of two or more of these main autoantibodies might strongly affect the risk of developing T1D (10–12); in fact, in children who develop autoantibodies against beta cells during the first 5 yr of life the risk of having clinically evident T1D in the following 10 yr progressively increase according to the number of autoantibodies ranging from 12.7% if only one autoantibody is present, to 61.6% if there are two and to 79.1% if three autoantibodies are detected. In contrast, if no autoantibody is observed, the risk is strongly decrease (0.4%) (Fig. 4) (12). However, research on humoral immunity in T1D is constantly evolving and the combined measurement of the different autoantibodies available might improve the ability to predict the onset of the disease in the very next future (10–13).

Anti-insulin antibodies (IAA): Insulin is a peptide hormone produced by pancreatic beta cells and has an important pathogenic role as T1D autoantigens. IAA production seems to be characterized by some specific peculiarities. In fact, high IAA titer during the first years of life predisposes young children to a rapid course of T1D; in particular, IAA levels above 2000 nU/mL are almost exclusively described in children who develop the disease before 5 yr of age. In contrast, less than 50% of patients who develop T1D after 15 yr of age has measurable levels of IAA (10). Current knowledge suggests that T lymphocytes response to proinsulin/insulin results in autoreactive B lymphocytes activation
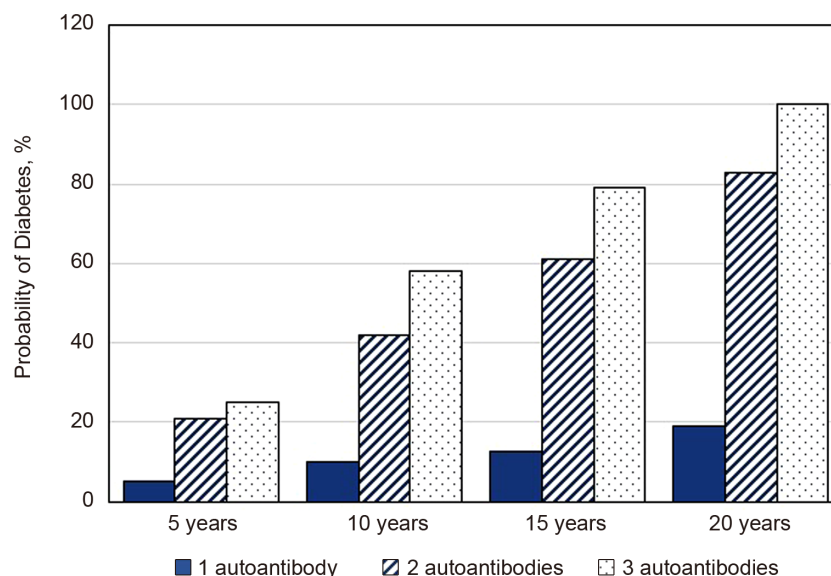
Fig. 4. Risk of developing T1D based on the number of antibodies against beta cells. Children who developed autoantibodies against beta cells in the first 5 yr of life showed a risk of having clinically evident T1D in the next 15 yr of 12.7% with only one autoantibody, 61.6% with two autoantibodies and 79.1% with three autoantibodies (with no autoantibody the risk is 0.4%) (12).

and production of autoantibodies against insulin (10). Studies in mice have clearly demonstrated that amino acids 9-23 in the B chain of the insulin molecule (the B:9-23), as well as the effects of 'processing' insulin in the beta cells, can determine the production of immunogenic epitopes (10). Numerous studies have been performed in patients with T1D that demonstrate a clear T lymphocyte response against proinsulin/insulin (including B:9-23), both in peripheral blood and in pancreatic lymph nodes (10). It has also been reported that CD4 lymphocytes isolated in the pancreas of patients with T1D respond to proinsulin epitopes restricted to HLA-DQ8 and to DQ8 transdimers that are formed in DQ8/DQ2 heterozygous individuals, thus emphasizing their role in the pathogenesis of T1D (14). Therefore, the characterization of IAA might be relevant during early childhood (15).

Anti-glutamic acid decarboxylase (GAD) antibodies: One of the first characterization of the presence of anti-GAD antibodies is reported at the end of the 80s when researchers by using a high-resolution 2-D electrophoresis revealed the specificity of a 64 K autoantibodies in serums of subjects with T1D. The nature of 64 kDa antigen remained unknown until it was reported that autoantibodies to GABAergic neurons and beta cells are present in an unusual condition called 'Stiff Person Syndrome'. Glutamic acid decarboxylase (GAD) is the enzyme that catalyzes the conversion of glutamic acid into gamma amino butyric acid (GABA), a potent inhibitory neurotransmitter; these observations subsequently led to the identification of GAD as the 64 kDa autoantigen in T1D. Anti-64 kDa antigen antibodies are present in 80% of newly diagnosed T1D patients and more importantly in individuals with prediabetes, already before the diagnosis of the disease (10). Therefore, antibodies against this antigen are useful in predicting the risk of developing T1D over a period of 11.5 yr in children and adolescents and in adults (10). The importance of anti-GAD antibodies in predicting the risk of developing T1D in children has recently been reiterated (4), showing their major role in the diagnostic criteria of the disease.

Anti-IA-2 antibodies (ICA512): The IA-2 neuroendocrine antigen (ICA512) is an important autoantigen in T1D. It is an enzymatically inactive member of the tyrosine-phosphatase family and is involved in the regulation of insulin secretion. In the characterization of the risk of developing T1D, anti-IA2 antibodies play a relevant role (10). The IA2 and its homologue IA-2ß (phogrin) are both neuroendocrine molecules. IA-2 is a protein of 979 amino acids with a single transmembrane region and a significant homology to the PTP receptor (RT-PTPase). Although, the extracellular part may also play an epitope function for the production of auto-antibodies, the major immunoreactive region of the IA-2 molecule is located in the amino acids region 601-979, which characterizes the intracellular domain of the protein (4). Recent studies have shown that the presence of anti-IA-2 results in more rapid progression of the clinically evident disease (10).

Anti-Zinc transporter antibodies (ZnT8): ZnT8 is a member of the cationic diffusion facilitator family with marked expression in several tissues and especially in pancreatic beta cells (4). Zinc is very concentrated in beta cells and it is essential for insulin storage. ZnT8 deletion in animal models induces glucose intolerance. In addition, it is associated to reduced beta cell zinc accumulation, to the production of insulin granule abnormalities and consequently to the reduction of insulin secretion after intravenous glucose loading and to increased levels of proinsulin (10). ZnT8 antibodies might be detected in 60–80% of patients with T1D, while they are found in less than 2% of healthy controls. In addition, ZnT8 antibodies are detected in less than 3% of patients with type 2 diabetes (T2D) and up to 30% of subjects with other autoimmune diseases associated with T1D (10). It is interesting to note that ZnT8 antibodies have been described in 26% of patients with T1D who did not have anti-GAD, anti-IA-2, IAA or ICA antibodies (4). There are numerous (even chimerical) variants of ZnT8, which have recently been used to screen subjects

at risk of developing T1D (10). Recent studies have confirmed the importance of the evaluation of ZnT8 antibodies, particularly during second and third childhood (4–16).

Prediction based on autoantibodies and metabolic markers

Many studies have confirmed that the presence of two or more autoantibodies against beta cell antigens has a high predictive value for the subsequent development of T1D with high sensitivity without significant loss of specificity (10–12). Furthermore, combining immunological and metabolic markers (by measuring early insulin response to glucose load with an oral glucose tolerance test [OGTT] or the frequent sample intravenous glucose tolerance testing [FSIVGTT]), might significantly improve the possibility of predicting the onset of T1D (11). Data shown by the Diabetes Autoimmunity Study in the Young (DAISY) have demonstrated that 89% of children who progressed to T1D had two or more auto-antibodies against beta cells (11); in particular, high titers of IAA and IA-2, but not of anti-GAD, significantly increase the risk of developing T1D during the course of pre-diabetes state in children with persistent high antibody levels (17). It is interesting to note that several studies have shown the paramount role of IAA particularly in young children (less than 5 yr of age). In fact, several studies have shown that the presence and especially the persistent detection of IAA in serum confers a risk of almost 100% of developing T1D after 5.6 yr. In addition, also fluctuating levels of IAA are associated with an increased risk, which however decrease up to 63% after 10 yr (7–10). The appearance of beta cell autoantibodies in the first years of life and with high levels of autoantibodies (in particular of IAA) is associated with a high risk of developing T1D in the following 7–10 yr (4–7). Therefore, the autoantibodies associated with T1D are the best and most reliable markers for the prediction of the disease and are also the first detectable and measurable signs of a pathological process

leading to the development of T1D. The presence of multiple islets autoantibodies is the marker of progression to clinically manifest disease, while the positivity for a single autoantibody would seem to detect a 'mild' autoimmune process, which most likely will not determine the onset of clinically manifest T1D (4–12). Although it is important to consider that rarely T1D can develop even in children with no islets autoantibodies, to date humoral immunity surrogate markers remain one of the most useful biomarkers in children and adolescents at increased risk of developing T1D. In addition, these markers represent the key characters to be evaluated in order to adopt primary and secondary prevention strategies (4, 5) with the aim of changing the natural history or eventually to cure T1D in children.

Prevention

There are about 550,000 children with T1D in the world and about 86,000 children are diagnosed with T1D every year. The incidence increases by about 3% per year and there are areas of the world (Finland, some areas of Sardinia, Canada, some regions of Sweden) where about one child out of 50 has T1D (18). T1D is one of the most frequent chronic disease in children and is associated to the risk of severe acute and chronic complications. This chronic disease is associated with relevant management issues and significantly negative effects on the quality of life of the children and their families. Therefore, massive human and financial resources have been continuously dedicating with the aim to adopt preventive strategies. The well-known natural history of the disease allows to plan different studies which strongly differ according to the design and the study population selection criteria as well as to the therapeutic approach. In fact, to date several primary prevention, secondary prevention and tertiary prevention studies have been concluded and a large number of trials are still ongoing. Particularly, available researches

have been focused on stopping immuno-mediated destruction of beta cells by adopting preventive, immunosuppressive and immunomodulatory treatments, either alone or in combination.

Primary prevention

Primary prevention is focused on asymptomatic children and adolescents at high risk for T1D according to the genetic predisposition defined by a positive family history or by the occurrence of known genes associated to T1D. Typical examples of primary prevention measures are to implement physical exercise to prevent childhood obesity or cardiovascular disease and to vaccinate children against infectious diseases (19).

A relevant study was the "Trial to Reduce Insulin-Dependent Diabetes Mellitus in the Genetically at Risk (TRIGR)" performed in infants with a first-degree relative with T1D and with the occurrence of high-risk HLA alleles. The first report from this study showed that in these children an exclusion diet (breast milk or hydrolyzed formulas compared to cow's milk) is associated to a 50% decrease of the risk of developing T1D related autoantibodies in the first 3 yr (20). Unfortunately, after 7 yr of follow-up this trial failed to demonstrate similar results showing no difference among the different diet options (20). Similar negative results were obtained in the "BABYDIET Study" in which a gluten free diet during the first year of life was not effective in reducing the production of autoantibodies and therefore the risk of developing T1D lifelong (10).

Vitamin D has also been used for primary prevention of T1D. However, although encouraging data have been obtained in small studies, such data have not been confirmed in larger and well controlled trials (21). Several trials have also failed in the primary prevention of T1D, either using specific antigens (insulin administration used parenterally [Diabetes Prevention Trial (DPT-1)], orally [DPT-1 orally], or intranasally [Diabetes Prediction and

Prevention (DIPP) intranasally], GAD) or using nonspecific antigens (such as diet, nicotinamide, decoso-hexanoic acid) (11). Some studies are currently underway with oral insulin (TrialNet), with GAD (Diamyd) or GAD + vitamin D3 or with drugs that regulate T lymphocytes (Teplizumab [anti-CD3], Abatacept [CTLA-4 Ig]), but results are not yet available (11).

Recent studies have confirmed the role of some enteroviruses (particularly some types of Coxsackie viruses) in determining T1D. These data support the interesting perspective to develop 'viral vaccine' against T1D (3), and now a large number of studies funded by the Juvenile Diabetes Research Foundation [JDRF] are ongoing, probably offering promising perspectives in the next future. As well, better results might be reached by new trials assessing the role of intestinal microbiota on immune regulation, and therefore on the risk of developing autoimmune diseases, including T1D (22): results are currently conflicting, but controlled studies are underway (DIPP, TEDDY) that will clarify the role of intestinal bacterial diversity in inducing the risk of developing T1D in children (22). Another preventive option is to use 'vaccines' based on 'tolerogenic' antigens that, by modifying immune regulation, are able to induce an immunological tolerance. The use of genetically modified cellular antigens for primary prevention also seems fascinating with the aim of strengthening T regulatory lymphocytes and to consequently modulate the immune mechanisms that underlie the beta-cell destruction (11).

Secondary prevention

Secondary prevention strategies are aimed at children and adolescents with two or more autoantibodies, with or without evidence of beta cell dysfunction. The "Diabetes-Prevention-Trial-1 (DPT-1)" was a North American trial aimed at assessing whether oral or parenteral insulin administration is able to prevent or delay the clinical onset of the disease; unfortunately, disappointing results were obtained (11).

However, in post-hoc analysis patients with higher IAA titers (greater than 80 nU/mL) had a significantly delayed onset of T1D when treated with oral insulin (4.8 yr); an even greater effect was observed in patients with even higher levels of IAA (higher than 300 nU/mL); these results justified the start of a new study with oral insulin (11). Another current study uses intranasal insulin (INIT-I and INIT-II). In INIT-I, intranasal insulin administration resulted in a reduction in T-cell response to insulin (11), while the INIT-II is still ongoing (11). GAD, another pancreatic islet autoantigen, could also represent a possible target of immune-mediated beta cell destruction. Although previous studies performed in newly diagnosed T1D patients did not provide the efficacy of GAD administration with an aluminum adjuvant (Diamyd®) (23), several studies are ongoing in subjects at risk (with Diamyd® alone or in association with high doses of vitamin D) (11) in order to further explore this hypothesis. An European Nicotinamide Diabetes Intervention Trial (ENDIT) has not shown any efficacy of nicotinamide (an antioxidant) in reducing the frequency of clinically evident T1D in children, adolescents and young adults with autoantibodies (11). More recently, in consideration of the good results obtained in patients with a new diagnosis (tertiary prevention) research has focused more on immunological modulation with monoclonal antibodies. Therefore, current studies with anti-CD3 antibodies (teplizumab and otelixizumab) and abatacept (CTLA4Ig) (which prevents CD28 from binding to its CD80 / CD86 ligands) might provide interesting results (24, 25).

Tertiary prevention

Larger data are available on tertiary prevention. In fact, most of the available prevention studies have been conducted in children, adolescents and adults with newly diagnosed T1D; with the aim of prolonging the remission phase (measured by characterizing endogenous insulin production [basal or

Table 1. Tertiary prevention trials in T1D

Results available	Ongoing studies	Ongoing enrollment
GAD	Mesenchymal stem cells	Lymphocytes TREG
*DiaPep277	GCSF	alpha-1 antitrypsin
*Anti-CD3 (Teplizumab)		
*Anti-CD20 (Rituximab)		
*CTLA-4 (Abatacept)		
*Autologous non-myeloablative transplantation		
*ATG-GCSF		
Mycophenolate Mofetil + anti-CD25		
IL-2 + Sirolimus		
Anti-CD2 (Alefasept)		
Canakinumab; Anakinra		
ATG		
Cord blood		
Cord blood + Vitamin D + Omega 3 fatty acids		
Meticulous metabolic control		

* indicates transient efficacy in obtaining better C- peptide levels, lengthening the remission phase and reducing insulin requirements in the first years of illness.

stimulated C-peptide] or by the need of exogenous insulin). Since the first studies with steroids or cyclosporine, the trials performed for tertiary prevention of T1D have however provided important information also on the pathophysiology of the disease and have laid the rational basis for the secondary prevention and primary prevention trials. Table 1 summarizes the most important tertiary prevention trials conducted in recent years (11). Some attempts with autoantigens (GAD alone or in combination, also by intralymphatic route) or with monoclonal antibodies (teplizumab, otelixizumab, rituximab, abatacept) have shown good clinical results in a first phase with transient preservation of C-peptide and brief metabolic improvement. However, after this phase the beta cell activity declines and patients become dependent on exogenous insulin administration (11). A Brazilian study of 23 patients led to (transient) insulin independence using autologous non-myeloablative stem cells transplantation, cyclophosphamide and granulocyte colony-stimulating factor (GCSF) (11). Such an aggressive approach was criticized very much

because of numerous side effects (26). Currently a study with GCSF is ongoing on a large number of newly diagnosed T1D patients (11).

New perspectives in T1D prevention therapies

Although, over the past 30 years great efforts have been made to identify new agents to prevent and avoid the onset of T1D, no therapy has been shown to be effective at preventing T1D in susceptible individuals or in patients with autoantibodies or at avoiding insulin dependence in newly diagnosed children. The best and most precise knowledge of the molecular mechanisms of T1D pathogenesis allows to look with greater optimism to the future, with the important target of preventing one of the worst chronic diseases in children (Table 2). Better knowledge of the molecular mechanisms of beta cell destruction could make it possible to characterize every single child and individualize therapeutic interventions; for example, since different lymphocyte subpopulations are involved in the mechanisms of cellular immunity, therapeutic opportunities may include strategies that

Table 2. Possibility of developing clinical research for the prevention of T1D (11)

Limits and needs	Actions to be taken
Limits in the knowledge on pathogenesis and on the natural history of T1D	Improve trial design and increase collaborative studies on the natural history of T1D
Need to identify better and more reliable biomarkers	Increase current efforts to create biobanks, using the latest technologies (systems biology)
Need to design better trials and to have better tests available to measure the obtained results	Use innovative trial designs and combined therapies in a large number of patients
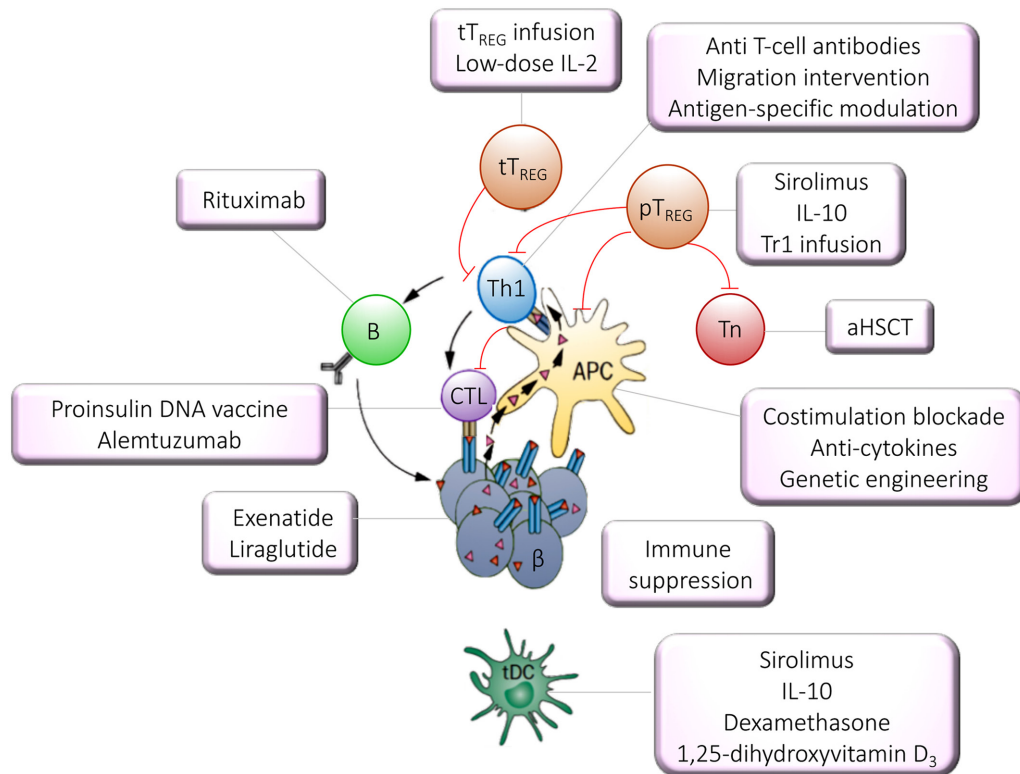
Fig. 5. New therapeutic strategies in T1D according to immunological modulation. Immune-pathogenetic mechanisms in T1D involve different leukocyte subsets. Autoreactive T lymphocytes are activated (TH1) by the beta-cell antigen presentation due to antigen-presenting cells (APC-dendritic cells [APCs], macrophages). The activation of autoreactive TH1 induce the autoantibodies production against beta cells by B lymphocytes and cytotoxic lymphocytes (CTL) activation, thus resulting in beta cells destruction. There are T regulatory lymphocytes (T_{REG}) and tolerogenic dendritic cells (tDC) able to suppress the autoimmune response specifically (pTREG cells) or non-specifically (tTREG cells). Novel treatments might be adopted with the aim of enhancing this "natural" regulatory system. aHSCT, autologous hematopoietic stem cells; APC, antigen presenting cells; CTL, cytotoxic T lymphocytes; pTREG, T lymphocytes peripheral regulators; tDC, tolerogenic dendritic cells; Tn, naive T lymphocytes; TREG, T regulatory lymphocytes; tTREG, thymic regulatory lymphocytes; β cell, beta cells; the autoantigens of the beta cells are represented by triangles (9).

enhance the 'natural' immunological regulation by leukocytes, as well as immunosuppression or immune depletion. Each molecule involved between beta cells and the immune system could be a 'target' of prevention treatment; many of these strategies are the subject of primary or secondary prevention studies and are summarized in Fig. 5 (9–27). Another important aspect is to differentiate the children who maintain a sufficient beta-cell mass (which could benefit from treatments that improve beta-cell function and protect the remaining beta-cell mass), from children with a high degree of inflammatory and autoimmune phenomena against beta cells (which could benefit from a specific immunological intervention) (9–28). Another promising approach is to improve the knowledge on the so-called 'trimolecular complex' consisting of T-cell receptor, autoantigen and HLA class II; in fact, from this interaction beta cell autoantibodies are produced. Several studies are ongoing with molecules that occupy a small pocket of the HLA molecule, inducing an inhibition in the presentation of the antigen (and subsequent activation of T lymphocytes) or an activation of a protective immune response (for example production of IL- 10). In other trials a monoclonal antibody that binds a specific peptide/HLA complex is used in order to block the activation of T lymphocytes. Finally, monoclonal antibodies against specific lymphocyte T (alpha or beta chain) receptors may be used to induce the depletion of autoreactive T lymphocytes (10–29, 30). The "INNODIA Project" (Translational approaches to disease modifying therapy of type 1 diabetes: an **INNO**vative approach towards understanding and arresting type 1 **DIA**betes) is a Consortium financed by the Horizon 2020 European Framework Program (31), which involves 26 academic institutions, 2 patient associations, 4 pharmaceutical industry partners (EFPIA) and small and medium size enterprise (SME). This consortium aims to improve knowledge on the molecular mechanisms that cause T1D in adults and children in order to use

innovative treatments for primary and secondary prevention of the disease (32). In fact, the greatest hope is that the basic research and clinical trials of this European Consortium, which, together with other Consortia in the USA (TrialNet, for example), could realize the objective and the dream of preventing T1D in children (24, 25).

Conclusions

Although new data have better defined the complex etiology underling the interrelation of genetic and environmental factors in the natural history of T1D, relevant pieces of the puzzle still are missing. The most recent progresses in this field pave the way for future studies, with the aim of preventing T1D in children. In the coming years data obtained by novel consortia will certainly provide interesting results and promising perspectives.

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